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Establishment of a Protocol for the Isolation of Feline Pancreatic Islets of Langerhans

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INDEX

SUMMARY.....4

ZUSAMMENFASSUNG DEUTSCH.....5

1. INTRODUCTION.....6 to 29

1.1. Feline diabetes mellitus

1.2. Pancreas anatomy, histology and function

1.2.1. Anatomy and histology

1.2.2. Function

i) Exocrine pancreas

ii) Endocrine pancreas

1.3. History of isolation of pancreatic islets of Langerhans

1.4. Procedure of islet isolation

1.4.1. Pancreas perfusion

i) Cold organ preservation

ii) Methods of perfusion

1.4.2. Pancreas digestion

i) Methods of digestion

ii) Enzymatic solutions

iii) Digestion temperature and time

1.4.3. Islet purification

1.5. Assessment of islet preparation

1.5.1. Morphology

1.5.2. Yield and size

1.5.3. Purity

1.5.4. Viability

1.5.5. Islet culture

1.5.6. Functionality

1.6. Islet isolation in cats

1.7. Aim of the study

2. MATERIALS AND METHODS.....30 to 38

2.1. Donor animals

2.2. Anatomical study in cats

2.3. Feline islet isolation

2.3.1. Pancreas perfusion

2.3.2. Pancreas digestion

2.3.3. Islet purification

2.4. Assessment of islet viability and quality

2.4.1. Viability

2.4.2. Morphology, yield and purity

2.5. Islet culture and functionality assessment

2.5.1. Glucose stimulated insulin secretion test (GSIS)

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

3. RESULTS.....39 to 51

3.1. Anatomical study in cats

3.2. Feline islet isolation

3.2.1. Pancreas perfusion

3.2.2. Pancreas digestion

i) Type of enzyme solution

ii) Size of the pancreas

iii) Time of digestion

iv) Mechanical disruption

3.2.3. Islet purification

3.3. Assessment of islet viability and quality

3.3.1. Viability

3.3.2. Morphology, yield and purity

3.4. Summary of achieved results

3.5. Islet culture and functionality assessment

4. DISCUSSION.....52 to 60

4.1. Pancreas perfusion

4.2. Pancreas digestion
4.3. Islet purification
4.4. Viability
4.5. Morphology, yield and purity
4.6. Islet culture and functionality
4.7. Conclusion

5. REFERENCES.....61 to 70

6. ANNEX.....71

7. LIST OF ABBREVIATIONS.....72

8. ACKNOWLEDGEMENTS

9. CURRICULUM VITAE

SUMMARY

Diabetes mellitus is a common endocrinopathy in cats; it is associated with lesions in the pancreatic islets e.g. islet amyloidosis and beta cell loss. Research on isolated islets greatly contributed to the understanding of the pathophysiology of diabetes in humans. Therefore, we aimed at improving the existing methods of isolation in order to increase islet yield, purity, viability and functionality of isolated islets in cats.

Nine cats were used as donors and islet isolation was successfully accomplished in eight cases. The isolation method that achieved the highest islet quality was characterized by the perfusion of the pancreas with 80ml of Collagenase type IV (Worthington) through the pancreatic duct at the site of the major papilla. The enzymatic digestion of the intact organ was combined with mechanical disruption and controlled by frequent dithizone staining. Purification was performed by a first filtration step followed by handpicking. Purified islets were then plated on extracellular matrix pre-coated plates (ECM) plates and cultured for 48h before a functionality test was performed.

For the first time, it was possible to isolate and culture feline islets with high degree of viability and purity. However, as the islet yield and the percentage of islet free from the acinar tissue relative to the total number of isolated islets was low compared to data on other species, further studies are required to improve the procedure of islet isolation in cats.

ZUSAMMENFASSUNG DEUTSCH

Wegen deren potentiellem Nutzen für die Erforschung der Pathophysiologie des Diabetes mellitus war es das Ziel der vorliegenden Untersuchung, eine verbesserte Methode zur Isolierung von Pankreas-Inseln bei Katzen zu etablieren, und zwar was deren Menge, Reinheit, langfristiges Kultivieren, und Funktionalität betrifft. Bei acht von neun Spender-Katzen war die Isolierung erfolgreich. Die Isolationsmethode, mit der die qualitativ besten Pankreasinseln isoliert wurden, beinhaltete eine Perfusion des Pankreas mit ca. 80 ml einer Kollagenase-Typ IV haltigen Lösung (Worthington) durch den Pankreaskanal, ausgehend von der grossen Papille im Dünndarm. Die enzymatische Verdauung wurde kombiniert mit einer mechanischen Zerkleinerung des Pankreas-Gewebes; kontrolliert wurde der Verdauungs-prozess durch häufige Anfärbungen mit Dithizon. Die Reinigung der Inseln erfolgte durch Filtration und einem manuellen Auslesen der besten Inseln. Diese gereinigten Inseln wurden auf mit extrazellulärer Matrix beschichtete Inkubationsplatten verbracht und für 48 h kultiviert, bevor funktionelle Tests durchgeführt wurden. Wir konnten zeigen, dass es möglich ist, lebensfähige und reine Insel-Kulturen bei Katzen anzulegen. Da allerdings die Gesamtzahl der von einer Katze isolierten Inseln und deren Reinheitsgrad im Vergleich zu anderen Tierarten gering war, sind weitergehende Untersuchungen notwendig, um die Isolierung von Pankreas-Inseln bei Katzen weiter zu verbessern.

1. INTRODUCTION

According to the *American Diabetes Association (2011)*, diabetes mellitus in humans is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or insulin action. Insulin is produced by the beta cells of the islets of Langerhans in the pancreas, and diabetes occurs when insulin secretion is impaired, or when tissues are resistant to the action of insulin, thereby compromising the body's ability to regulate glucose metabolism (Mooney and Peterson, 2012).

Nowadays, human Diabetes mellitus (DM) is classified based on the pathogenesis of beta cell failure in four different categories: type 1, type 2, gestational diabetes and other specific types of diabetes (Expert Committee, 1997). In veterinary medicine and namely in cats, the classification system follows the same criteria as in humans, although the etiopathogenic mechanisms of diabetes may differ in some aspects between the two species (Feldman et al., 2015).

In humans, type 1 Diabetes mellitus (T1DM) results from cell-mediated autoimmune damage to pancreatic beta cells leading to an absolute insulin deficiency (Feldman et al., 2015). Although rare in cats (Sparkes et al., 2015), clinical and histologic findings consistent with T1DM have been described in some diabetic cats in the literature (Rand, 2013; Nelson and Reusch, 2014).

The most common form of diabetes in humans (which accounts for ~90–95% of the cases; American Diabetes Association 2012) and cats (approximately 80%; Feldman et al., 2015) is type 2 diabetes mellitus (T2DM), a condition characterized by insulin resistance, defective insulin secretion, islet amyloid formation, and beta cell loss (Rand et al. 2004; American Diabetes Association, 2012). Cats are among the few species, besides humans and other primates that spontaneously develop this form of diabetes (Henson and O'Brien 2006).

The standard care for human T1DM today is still similar to what it was in the early 1920s, obviously with a much larger range of different insulin treatment options (e.g., fast-acting, slow-acting, injectable, and inhalable insulin). Despite countless efforts using animal models with T1DM that were treated with immune-modifying therapies, none of these has demonstrated comparable efficacy in humans. Hence, because metabolic control is far from ideal in many diabetics, there is an unmet need for better treatment options for T1DM that address all aspects of the disease (Rekers et al., 2015). Pancreatic islet allo-transplantation is a procedure in which islets from the pancreas of a deceased organ donor are purified, processed,

and transferred into another person. Pancreatic islet allo-transplantation is currently labeled an experimental procedure until the transplantation technology is considered successful enough to be labeled therapeutic. It is performed in certain patients with T1DM whose blood glucose levels are difficult to control (http://www.niddk.nih.gov/health-information/health-topics/Diabetes/pancreatic-islet-transplantation/Pages/index.aspx).

Islet cell transplantation is also being tested as a future option for treating type 2 diabetes (http://www.niddk.nih.gov/health-information/health-topics/Diabetes/pancreatic-islet-transplantation/Pages/index.aspx).

1.1. Feline diabetes mellitus

The prevalence of feline diabetes mellitus (FDM) has risen in the last decades possibly because of the increase of risk factors such as obesity, physical inactivity and increased average life expectancy (Guptill et al., 2003; Reusch, 2011; Ohlund et al., 2015). The incidence of FDM may range from one in 50 to one in 400, depending on the population studied (Henson and O'Brien, 2006).

When the responsiveness of the body to the effects of insulin in promoting glucose disposal, known as insulin sensitivity, is impaired, insulin secretion is increased to prevent that blood glucose concentration increases. In T2DM, however, the compensatory increase in insulin secretion fails to compensate for the demand of insulin and results in hyperplasia and hypertrophy of beta cells ultimately leading to beta cell dysfunction and failure (Rand, 2013).

The resultant hyperglycemia provokes insulin resistance in peripheral tissues and a chronic high demand to secrete insulin which further exacerbates beta cell failure and induces beta-cell loss through apoptosis – a state known as beta cell exhaustion (Rand and Marshall, 2005).

It is currently accepted that obesity and other insulin-resistant states act similarly to increase the need for insulin secretion by beta cells, which cannot be met in individuals whose beta cells are damaged by some other disease process.

Theories about the cause of this failure of compensation have included 1) damage to pancreatic islets by amyloid deposition and a variation of the amyloid hypothesis called the toxic oligomer hypothesis; 2) toxicity by glucose, lipids, or both; 3) reactive oxygen species; and 4) inflammatory cytokines (Rand, 2013).

Once diagnosed, the appropriate initial treatment must start as soon as possible. A reduction of the hyperglycemia and hyperlipidemia may increase the chances to preserve the function of pancreatic beta cells and the remission of diabetes (Rand and Marshall, 2005). The initial

treatment of uncomplicated diabetes in cats usually consists in the administration of insulin and/or oral hypoglycemic agents, and by use of dietary modification (Mooney and Peterson, 2012).

1.2. Pancreas anatomy, histology and function

1.2.1. Anatomy and histology

The pancreas is the second largest gland of the body (Samuelson, 2007).

It is a pale, lobulated organ (Hudson and Hamilton, 2010), located in the craniodorsal part of the abdomen in close association with the duodenum. It has a U-shaped form (O'Brien et al., 1986) and can be divided into three parts: body, left and right lobe (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology). It develops as a compound, tubuloalveolar gland and is both an exocrine and an endocrine organ (Banks, 1986).

A thin capsule of connective tissue covers the pancreas and sends septa into it, separating the pancreatic lobules (Mescher, 2010).

The quantity of connective tissue of the gland varies between species and is comparably small in cats. The size and shape of the lobules vary between individuals of the same specie and in what concerns to cats, lobules are small and quite distinct (Harris and Gow, 1893).

The interlobular connective tissue contains blood vessels, lymphatics and nerves (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology).

The splenic, hepatic and superior mesenteric arteries send the coeliac and cranial mesenteric as branches to the pancreas for its blood supply. Vessels arborize in the connective tissue septa and create a rich capillary network that surrounds each acinus and invades each islet. The endothelium of capillaries in the exocrine pancreas is continuous, whereas the one surrounding the islets is fenestrated. Islets receive ample blood supply to enable an appropriate response to blood glucose level (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology). Venous blood is collected by several tributaries to the hepatic portal vein (Hudson and Hamilton, 2010) that run between the left and right lobes (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology). Lymph from the pancreas drains into the pancreatic duodenal lymph nodes. Small lymphatic vessels drain into larger ones that follow the course of the blood vessels in the connective tissue (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology).

The pancreas receives sympathetic and parasympathetic supply (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology). Nerve terminals adjacent to

islets and synaptic endings are seen only rarely in cats (Wieczorek et al., 1998). In general, nervous regulation seems to be less important than the hormonal control in pancreas (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology).

1.2.2. Function

i) Exocrine pancreas

The exocrine pancreas consists of numerous lobular secretory acini, surrounded by a basal lamina and ducts, which are held together and supported by reticular fibers and a rich capillary network (Mescher, 2010). It is responsible for the synthesis, storing and secretion of digestive enzymes such as proteases, lipases and amylase (Wieczorek et al., 1998) that, together with the digestive enzymes produced by other glands of the digestive system, facilitate the breakdown of ingesta (Samuelson, 2007).

The lumen of the *acini* drains into intercalated ducts, which in turn converge into larger interlobular ducts. The latter converge to interlobar ducts that can be found in the connective tissue septa between lobules. Interlobar ducts join to form either the pancreatic or the accessory duct that drain into the duodenum. In some species such as cats, the pancreatic duct unites with the bile duct and bile and pancreatic juice enter the duodenum together (https://en.wikivet.net/Pancreas_-_Anatomy_%26_Physiology).

ii) Endocrine pancreas

"….This cell has a small and polygonal structure. Its cytoplasm is perfectly brilliant and free from granules, with a distinct nucleus that is round and of discrete dimension. This type of cell clusters, generally in large number, is diffusely scattered in the glandular parenchyma. These clusters have generally a diameter of 0.1-0.24 mm and may be easily distinguished in fragmented pancreatic preparations…". These were the words used by the German anatomist and anthropologist Paul Langerhans to describe pancreatic islets in his dissertation published in 1869 (Langerhans, 1869).

The islets of Langerhans are structures unique to vertebrates, which are embedded in the exocrine tissues. They are the endocrine part of the pancreas representing approximately one to two per cent of the pancreatic mass in adults (Wieczorek et al., 1998). Apparently there is no difference in number of pancreatic islets between the three-pancreatic regions (Lutz and

Rand, 1997). Occasionally, islets are also found in the interlobular connective tissue in proximity of an interlobular pancreatic duct (Maeno et al., 2006).

Pancreatic islets are highly vascularized structures, which consist of several hundred endocrine cells that function together to maintain glucose homeostasis (Steiner et al., 2010). The islets are surrounded by collagen and reticulin fibers, which in general are not considered a real capsule (Wieczorek et al., 1998). Contrary to what happens in other species, the basement membrane-like material surrounding the islets is very scarce in cats. Thus, acinar cells appear to be in direct contact with endocrine cells, which can be distinguished by their characteristic granules (Maeno et al., 2006).

The average diameter of pancreatic islets is 100-200μm but some are much smaller consisting of only a few cells. The size and arrangement of cells within each islet vary according to the location within the pancreas, age, specie (Samuelson, 2007) and even within individuals of the same species (Steiner et al., 2010). Moreover, islet structure changes in response to physiological or pathological stimuli, such as those induced by diabetes mellitus. In mice, for example, conditions such as pregnancy and obesity result in increased beta cell mass and islet size accompanied with architectural changes (Steiner et al., 2010).

In most domestic animals, the clusters of endocrine cells consist of four cell types based on a combination of morphological and histochemical features, each secreting different hormones (Samuelson, 2007):

- α or Alpha cells, form the second-most numerous type of endocrine cell within the islet. In cats, α cells are often found in large clusters, centrally or ecccentrallly located in the islets. Solitary or small clusters (2 to 3 cells) of α cells were also detected around the periphery of islets (O'Brien et al., 1986). These cells are involved in glucagon production, which promotes glucose release from the liver, namely when blood glucose levels are low (Samuelson, 2007).
- β or Beta cells, comprise the largest population of secretory cells within the pancreatic islets. They are arranged in compact cords usually 2 to 3 cells wide and bounded by thin fibrovascular septa (O'Brien et al., 1986). These cells form and release granules filled with insulin and amylin.

  When secreted, insulin lowers blood glucose levels primarily through the cellular uptake of glucose (especially by skeletal muscle fibers and adipose tissue) and inhibiting the release of glucose from the liver (Samuelson, 2007).

Amylin is co-secreted from the beta cells with insulin. In feline diabetes mellitus, amylin-derived amyloid can be found in pancreatic islets. This deposition increases with age, but is significantly greater in cats with impaired glucose tolerance or overt diabetes, compared with age-matched cats with normal glucose tolerance (Lutz et al., 1994).

- δ or D cells, form another small population of cells within the pancreatic islet. In cats, solitary D cells, or sometimes small clusters (2 to 3 cells) of D cells, are scattered throughout the islets between β cell cords and at the islet periphery (O'Brien et al., 1986). These cells secrete somatostatin, which is able to inhibit the release of glucagon and insulin by α and β cells, respectively (Samuelson, 2007).
- PP cells secrete pancreatic polypeptide (PP). The exact physiological role of PP cells is unknown.

A 5th type of cells is represented by a relatively small portion of cells: the C cells. They are mostly young developing cells that will eventually mature into specific secretory cells within the islet (Samuelson, 2007).

In cats, islets in all 3 regions (right lobe, middle region and left lobe) of the pancreas appear randomly distributed within exocrine lobules. Occasionally, islets are found in the interlobular connective tissue, usually in the vicinity of an interlobular pancreatic duct (O'Brien et al., 1986).

As reported in other species, the mean number for each cell type in cats decreases in this order: β, α, δ, and PP-cells (O'Brien et al., 1986). The relative number of α, β and δ cells is similar to the ones described in humans. Interlobular islets have a centralized area of α cells with an equal number of surrounding β cells and a few solitary δ cells (Steiner et al., 2010).

1.3. History of isolation of pancreatic islets of Langerhans

An association between pancreas and clinical diabetes was made for the first time in 1788 by Thomas Cawley who described pathological anomalies in the pancreas of diabetic individuals (Cawley, 1788). Similar findings were confirmed by others (Bouchardat, 1875; Lancereaux 1877; Rolleston, 1934) and in 1889, Mering and Minkowski showed that diabetes could be induced by the removal of the pancreas (Luft, 1989). Few years later, Schäfer (1895) suggested a role for the islets of Langerhans in the prevention of diabetes and attributed the specific name “Insulin”, which comes from the Latin insula (meaning island), to the hormone

produced by the islets (Downing, 1983). In 1921, Banting and Best first extracted insulin from dogs' pancreas and then administered it to diabetic dogs lowering their abnormally high blood sugar (Banting and Best, 1922; Bliss, 2007).
The first attempts to transplant pancreatic fragments began well before the discovery of insulin and demonstrated that the transplantation of pancreatic fragments into diabetic animals could cure the disease (Minkowski, 1892; Williams, 1894). The idea to physically separate the endocrine from the exocrine components of the pancreas was originally proposed by Leonid W. Ssbolew in 1902 (Ssbolew, 1902), but was pursued only 60 years later when Hellerstroem (1964) described for the first time a protocol for the isolation of the islets of Langerhans in mice.

Originally, pancreatic islet isolation consisted of a technique of microdissection of the organ under the microscope. However, both the yield and the quality of the isolated islets were not satisfactory. A new phase begun in 1965 when Moskalewski described the effect of collagenases on pancreatic fragments and the enzyme was added to the isolation process (Moskalewski, 1965). In 1967, Lacy and Kostianovsky improved this technique by introducing the perfusion of the pancreas via the pancreatic duct and by employing sucrose gradients to help purify the digestate (Lacy and Kostianovsky, 1967). Subsequently, Arnold Lindall and Coll at the University of Minnesota used a discontinuous gradient solution (Ficoll) to achieve higher yields of islets, although, initially, islets were not functional (Lindall et al., 1969). Only after Ficoll was dialyzed and lyophilized, vital islets could be obtained for experimental transplant studies. The process of islet cell isolation established by Lacy (Lacy and Kostianovsky, 1967) consisted of two phases: (i) islet cluster dissociation and dispersion (ii) islet purification from the pancreas. This technique became the gold standard for rodent islet isolation allowing the accomplishment of several important studies.

In large animals, however, Lacy's protocol did not lead to similar results and large parts of the islets were lost during the purification process. In 1981, Atsushi Horaguchi and Merrell established a new protocol for the isolation of islets in dogs consisting of three phases: (i) the cannulation of the pancreatic duct with intra-ductal injection of collagenase solution to better digest the fibrotic structures; (ii) mechanical dissociation with digestion at 37°C; and (iii) filtration of the pancreatic digest through a 400mm filter mesh. With this approach, islet recovery was estimated to 57% with a purification of approximately 10%; hence, it became

possible to obtain adequate numbers of islets for transplantation from a single donor (Horaguchi and Merrel, 1981).

In the 1980's, a new islet isolation technique was adopted in dogs and humans that consisted of intraductal injection of collagenase, dispersion of the pancreatic tissue by mechanical agitation and passages through a series of graded needles followed by purification using filtration and centrifugation on density gradient solutions. This method resulted in a higher degree of purity of approximately 20-40% (Gray et al., 1984; Alejandro et al., 1987). Modifications of the procedure improved purity further and allowed the first transplantations of pancreatic islets in dogs (Warnock et al., 1988; Catrall et al., 1989).

A turning point in the history of islet isolation was reached in 1988 with the introduction of an automated method for the control of pancreas digestion. This method consisted of a dissociation chamber (Ricordi chamber) in which the pancreatic tissue was progressively digested and the released material was continuously collected in a separate solution designed to inhibit overdigestion (Ricordi et al., 1988). This method dramatically increased islet cell yield and purity and has represented, ever since, the gold standard for the isolation of islets in human (Ricordi, 2003) and large animals (Ricordi et al., 1990).

1.4. Procedure of islet isolation

A classical procedure of islet isolation includes three main steps: i) pancreas perfusion, ii) pancreas digestion and iii) islet purification (Li et al., 2009).

1.4.1. Pancreas perfusion

i) Cold organ preservation

In humans, it is standard practice to cannulate the abdominal arteries to allow intravascular infusion of a hypothermic preservation solution in the transplant donor. Cold organ preservation prior to the perfusion has the advantages of decreasing the rate of cell deterioration in the organ and of reducing the metabolic activity of the pancreatic tissue (Vrabelova et al., 2014). In animals, it is especially advantageous when it is not possible to perfuse the pancreas immediately after euthanasia.

ii) Methods of perfusion

The first step of the isolation process involves the introduction of the enzyme solution into the pancreas. Many different perfusion techniques have been described and tested but it seems

that the technique needs to be chosen according to the characteristics and size of the pancreas in the different species (Burghen and Murrel, 1989; O'Gorman et al., 2005).

In rodents, three main techniques are usually applied: i) the pancreas is excised from an euthanized animal, cut into pieces and immersed into the enzymatic solution, thus increasing the surface area and providing conditions for the digestive enzyme to break down the tissue surrounding the islets (Lacy and Kostianovsky, 1967; O'Dowd 2009); ii) the enzyme is injected into the common bile duct (CBD), the pancreas is then excised and digested without being cut into pieces (Gotoh et al., 1985); iii) the enzyme solution is injected directly into the lobes of the pancreas through the capsule; the latter method may be applied when the bile duct is compromised or cannot be used (Carter et al., 2009).

In humans, pancreatectomy is usually performed prior to the cannulation procedure. Then, one or more catheters are inserted into the main pancreatic duct, either in the middle or at the extremities of the body. A small amount of solution (e.g., Hanks' solution) is often injected prior to the enzymatic perfusion in order to help identifying the course of the main duct. The pancreatic parenchyma is then minimally cut at the mid-body and the main duct exposed. As an alternative to catheters, a polyethylene tube is inserted into the duct from the head of the pancreas and advances to the tail. The injection of the enzyme solution distends the tail portion first and when the tube is pulled back to the head, the solution may efficiently distend the whole pancreas (Seiji et al., 1999; Kin and Shapiro, 2010).
All approaches described above are currently used in rodents and humans but comparisons in respect to isolation efficiency and islet purity are difficult since considerable variations are present among the published methods and adaptations have been made based on species-specific characteristics.

In summary, it is generally accepted in all species that the perfusion of the pancreas through the pancreatic duct, using anatomical structures, improves the enzyme diffusion into pancreatic *acini* and consequently induces a more selective and effective digestion of the exocrine pancreas (Zini et al., 2009). The main limitation of the method is that considerable experience and expertise are required to locate and cannulate the common bile or the pancreatic duct (Li et al., 2009).

1.4.2. Pancreas digestion

i) Methods of digestion

The enzymatic dissociation of the endocrine tissue from the surrounding acinar tissue is a critical step of the islet isolation process (Agarwal and Brayman, 2012).

In general, two main methods are used to perform an enzymatic digestion:

a) The manual method is mostly used in rodents, cats and caprines and consists of the incubation of the entire pancreas or pieces of it in the enzyme solution placed in a water-bath.

b) The automated or Ricordi's method consists of a tissue dissociation chamber with a system that allows the recirculation of enzyme solution. The pancreas is placed inside a stainless steel chamber and mechanically dissociated by gentle agitation. Minimal physical trauma is caused to the islets, which are collected as they are liberated from the digestion chamber.

Both methods of digestion have been applied with success in pigs and dogs (Jiang et al., 2012; Jin et al., 2014; Vrabelova et al., 2014; Woolcot et al., 2012).

Pancreas digestion can be influenced by several factors, which include the route of administration of the enzyme solution, the type, concentration and activity of the enzyme and the digestion time and temperature. Digestion is completed when the action of the enzyme is stopped, which is usually achieved by a combination of cooling and removal of the enzyme from the tissue (Islam, 2015).

ii) Enzymatic solutions

Collagens are the major fibrous proteins that constitute the islet–exocrine interface. As a result of their dense structure and mechanical strength, collagens are not generally degraded by common proteases but can be degraded with high specificity by collagenases. As such, collagenase is a key component of the enzymatic solution used to isolate pancreatic islets (Islam, 2015) in humans and experimental animals (Zini et al., 2009). Many different types of collagenases are available and the advantages and disadvantages of each formulation were studied in several comparative studies (Caballero-Corbalán et al., 2009; Barnett et al., 2005; Yesil et al., 2009; Wang et al., 2011; Shimoda et al., 2010).

In the 1960s collagenase derived from Clostridium histolyticum became commercially available and known as "crude collagenase". C. histolyticum could be cultured in large

quantities in simple media and produced a wide variety of collagenases / proteases, which degrade various types of collagen and gelatin. The initial characterization identified six principle isoforms grouped into class I and II, based on substrate specificity and amino acid analysis. Another important characteristic was that binding of clostridial collagenase to collagen was not inhibited by low temperature, unlike enzyme activity in general (Kin et al., 2007).

The preparations available at the time were, however, very heterogeneous containing several other proteases and enzymes in addition to collagenase (Kin et al., 2007), cellular debris, pigments and endotoxins (Hyder, 2005). Therefore, both composition and activity of crude preparations were not consistent between different batches. This variability was recognized as the major obstacle for successful islet isolation in humans.

For these reasons, a novel collagenase blend, Liberase HI (Roche Applied Science, Indianapolis, IN), containing class I, class II collagenases and thermolysin, was introduced in the market. This highly purified enzyme formulation presented a reduced lot-to-lot variability and a low-endotoxin content. The use of Liberase improved the yield, function and viability of isolated islets in human and animal models compared to the historical use of crude preparations from C. histolyticum (Kin et al., 2007). Its use also resulted in shorter digestion times and less fragmentation of islets (Paget et al., 2007; Hyder, 2005).

However, in early 2007, the potential risk of prion disease transmission with the use of Liberase was reported; this was due to the presence of a bovine neural component in the manufacturing of the product. Moreover, significant lot-to-lot and even intra-lot variability were still present.

After the discontinuation of the manufacturing Liberase HI, Roche Diagnostics (Indianapolis, IN) developed a new enzyme product: Liberase MTF (mammalian tissue free), which was similar to Liberase HI with the exception that no mammalian tissue was used in the manufacture of the collagenase component. MTF proved that it could be successfully used for high-yield human islet isolation and clinical transplantation (O´Gorman et al., 2010).

Comparative studies between Liberase HI and Collagenase NB1 (SERVA, Heingelberg, Germany) were also performed (Brandhorst et al., 2010) and reported no differences with respect to pre-purification, post-purification, post-culture islet mass and islet yield between

the two enzymes, even though isolation obtained with Liberase HI resulted in significantly more viable islets. Overall, Serva collagenase NB1, a mammalian-tissue free enzyme blend (Iglesias et al., 2012), was considered another alternative to Liberase HI.

Today, other commercially available products, such as the Vitacyte collagenase HA, have demonstrated digestive capabilities comparable to those of the Serva Collagenase NB1. In a comparative study between the collagenases from Roche (Liberase MTF), Serva (Collagenase NB1), and VitaCyte (CIzyme Collagenase HA) (Indianapolis, IN), the latter presented the highest collagen degradation activity (Islam, 2015), as well as a much lower time needed for digestion (when compared to Serva NB1), without deteriorating islet integrity; this facilitated a more cost-effective isolation since it uses a less amount of enzyme (Caballero-Corbalán et al., 2009).

Independently of the specific characteristics of each product, enzyme blends with high purity and precise composition are required to ensure consistent activity and reproducibility of islet isolation in humans (Seiji et al., 1999). In rodents, the use of less expensive, crude or partially purified preparations is a common practice, even though this results in higher lot-to-lot variations in activity, in the consistency of islet yield and quality, requiring adjustments to the dose of enzyme used prior to the experiment.

Another important issue that seems to play an important role in collagen degradation is the class I to class II collagenase ratio (Barnett et al., 2005).
These two classes of collagenases, purified from *C. histolyticum*, compose the modern enzymatic preparations. They exhibit differences in their amino acid sequences and secondary structures, but show very similar catalytic domains. Collagenase I attacks residues near the ends of triple helix collagen domains (Yesil et al., 2009), is more stable and has a greater activity toward insoluble collagen (Barnett et al., 2005). Collagenase II digests at more interior sites (Yesil et al., 2009) and has a more broad specificity characterized by the ability to attack various peptide substrates at a much greater rate than class I (Barnett et al., 2005).
Some studies (Van Wart and Steinbrink, 1985; Wolters et al., 1995) have shown a synergistic effect on collagen degradation when both class I and class II activities were present. However, there is still some ambiguity about the specific role of each of the classes of collagenases on islet isolation and also about the optimal ratio between the two classes (de Haan et al., 2004).

The use of purified collagenase alone may, in some cases, not be enough for an adequate tissue digestion, resulting in a small islet yield; the presence of non-collagenolytic enzymes, such as Neutral Protease, may therefore sometimes be required to enhance collagenase-induced dissociation (Kin and Shapiro, 2010).
The protease to collagenase ratio has also to be carefully adjusted in a narrow range and according to the characteristics of the donor pancreas. In fact, excessive exposure to non-collagenolytic enzymes may provoke islet fragmentation and disintegration resulting in reduced islet viability. The effect of proteolysis may also lead to a decrease in collagenase activity throughout the isolation process (Kin et al., 2007).

iii) Digestion temperature and time

Today, *pancreata* are usually digested at 37°C in all species since limited evidence supports the advantages of lower digestion temperatures with respect to islet yield and viability (Kin et al. 2007).

Digestion time varies among different protocols based on the method selected for the cannulation of the pancreas. For instance, the perfusion of collagenase via the pancreatic duct allows the most direct access of collagenase to the collagen around the islets, which shortens the digestion time compared to other perfusion methods. Strain- and age-related changes in connective tissue can also influence the time of digestion (Islam, 2015). For example, pancreatic fibrosis may increase the duration of pancreas digestion while lipomatosis (characterized by the replacement of exocrine pancreatic parenchyma with adipose tissue or fat infiltration), may accelerate pancreas dissociation during islet isolation (Kin and Shapiro, 2010).
In general, digestion times should be kept as short as possible in order to limit the stress to the islets induced by the exposure to the enzymes and to reduce the possibility of contamination with exocrine tissue. In fact, exocrine tissue secretes digestive and in particular proteolytic enzymes that may compromise islet integrity and viability (Yesil et al., 2009).

1.4.3. Islet purification

At the end of the digestion step, the pancreatic digestate contains both endocrine and exocrine cells. The purification of the islets from the exocrine tissue is an important step for the isolation of pure islets especially because elimination of acinar cells prevents that their

digestive enzymes and proteases (e.g., gastrin-releasing peptide, amylase) decrease islets survival, integrity and functionality (Carter et al., 2009).
In some protocols, a filtration step through stainless steel filters (500μm) may sometimes be applied before purification. This step allows the digested tissue to be separated from the non-digested tissue, fat and lymph tissue, before is further purified in the subsequent step (Islam, 2015).

Final purification can then be achieved by four different methods: (a) sedimentation, (b) filtration, (c) gradient separation and (d) magnetic retraction (Li et al., 2009).

a) The sedimentation method is the easiest and less expensive, although it is also the most inefficient one. The major limitation is that islets normally show intrinsic variation in diameter (50–500μm), which can overlap with acinar cell diameters so that the separation is not very efficient (Islam, 2015).

b) In humans and large animals, filtration of pancreatic digestate does not provide a satisfactory degree of purity, contrary to what happens in rodents (Li et al., 2009). The yield of islets obtained with the filtration method is also lower compared to other methods (Ramirez-Dominguez and Castaño, 2015).

c) The density gradient or isopycnic centrifugation is based on the principle that, during centrifugation, tissue migrates and settles to the density that is equal to its own density. Using this technique, separation can be achieved based on intrinsic differences in density between islet tissue (~1.059g/mL) and exocrine tissue (1.059–1.074g/mL). Various gradient media have been developed and tested for islet purification. One of the most commonly used media is a synthetic polymer of sucrose-based media (Ficoll; Amersham, Uppsala, Sweden) (Islam, 2015). Other density gradient solutions such as the hypertonic solution Histopaque (Sigma, St Louis, USA) and the iodixanol (OptiPrep), a non-ionic, iso-osmolar solution, have been reported to yield successful clinical outcomes in islet transplant studies and to significantly reduce the secretion of proinflammatory cytokine/chemokines from the islet, in comparison to Ficoll-based density gradients (Mita et al., 2010).

Another new gradient medium, a mixture of University of Wisconsin (UW) and Ficoll-sodium- diatrizoate (Biocoll; Biochrom, Berlin, Germany) has also been used for density gradient separation (Huang et al. 2004) and showed improved post-purification islet yield when compared with the standard Ficoll medium.

The purification of large numbers of human islets has advanced rapidly with the introduction of the COBE 2991 cell processor (COBE Laboratories Inc., Lakewood, CO, USA) with Ficoll density gradient (Islam, 2015). This semi-automated computerized cell processor, originally developed for producing blood cell concentrates, is now the gold standard for islet cell purification and an indispensable equipment in human islet processing facilities.

d) Magnetic retraction: in rats, intra-arterial infusion of iron-oxide is used as an alternative to the filtration technique. The iron particles are preferentially entrapped in the islet capillaries; after digestion with collagenase, the iron-loaded islets are collected with magnetic retraction (MR) (Töns et al., 2008). In a comparative study in rats, MR yielded more pure, viable and functional islets than the conventional method of density-gradient purification method (Pinkse et al., 2004).

In some protocols a second purification step by gradient, sedimentation or filtration is needed before culturing, to further increase islet purity and islet yield, particularly if the volume of packed tissue is still large (Islam, 2015).

The handpicking of the islets before culture is also considered in some protocols as further purification step. Islets are manually selected from the acinar tissue and transferred to a second culture medium or third dish (depending on the initial purity of the preparation) containing culture media. It is a time consuming technique, and should be performed as fast as possible in order to minimize the time islets are outside the sterile hood and incubator and to limit the exposure to contamination and pH changes.

1.5. Assessment of islet preparation

It is of great importance that the isolation procedure yields islets of sufficient quality and that the assessment of the quality is performed according to standardized criteria. This has proven to be very challenging and standardization is lacking among different laboratories.

1.5.1. Morphology

The first assessment of islet quality is done by visual inspection of their morphology and provides basic information regarding their general health. Under the light microscope, rodent healthy islets appear spherical and golden-brown with a variable size between 50 to over 400μm (Carter et al., 2009) and are surrounded by a well-preserved capsule (Islam, 2015).

1.5.2. Yield and size

The total number of islets obtained after the isolation depends on several variables that are mostly related to the specific characteristic of the donor (age, weight, and strain), the genetic background (in rodents) (Jin et al., 2014), the isolation method and the experience of the operator in performing the isolation (Islam, 2015).

Quantification of the yield in an islet preparation can be performed by handpicking of previously stained islets under a light microscope. Islet can be specifically stained by the zinc-specific binding dye dithizone (DTZ) that binds zinc ions contained in beta cells, staining the islets in red.

The standard estimate of the volume of isolated islets is referred to a standard diameter of 150μm and which is defined as Islet Equivalent (IEQ). Islets of varying diameters are normalized to IEQs by mathematically compensating for their volumes. Quantification of islets with this method is however not generally accepted and in some cases, the absolute number of islets is used as measure of islets yield (Islam, 2015).

Large islets are commonly defined as islets with a diameter >150μm and small islets with a diameter <150μm; islets with a diameter lower than 50μm are usually excluded from the quantification (Hani et al., 2010; Vakhshiteh et al., 2013). Small-sized islets are reported to be more abundant in many species, including humans, pigs, and goats (Vakhshiteh et al., 2013) and to be superior to large islets with regard to *in vitro* insulin secretion and survival rate during both normoxic and hypoxic culture conditions (Lehmann et al., 2007; Vakhshiteh et al., 2013).

In mice, islet yield ranges from 200–400 with average yields of 300 islets per mouse. In rats, yields are of approximately of 600 to 800 islets per animal. In humans, although the pancreas is thought to contain over one million islets, islet isolation typically yields approximately 250,000–450,000 islets (Carter et al., 2009).

1.5.3. Purity

Purity is another key factor, which defines the quality of an islet preparation (Islam, 2015). It is defined as the percentage of free and the percentage of islets trapped into exocrine tissue to the total number of islets counted; purity is determined by visual inspection of a representative sample of the islet preparation (Standard operating procedure of the NIH clinical islet transplantation consortium, 2015).

DTZ staining is commonly used to help the assessment of purity, size and shape of the islets in several species like caprines (Hani et al., 2010) and pigs (Loganathan et al., 2014; Alexander et al., 2014). Immunohistochemical staining (such as immunoperoxidase technique) may also be used to assess the degree of purity of the final preparation (Hani et al., 2010).

1.5.4. Viability

Viability is generally defined as the ratio of healthy living cells to dead or dying cells and is assessed using cell exclusion or DNA-binding dyes (Carter et al., 2009).

Islets are aggregates of cells, therefore, it is expected that some cells within an individual islet will be alive and some may be dead due to the isolation procedure. The goal of the viability test should be to assess, as accurately as possible, the percentage of islet cells that is viable within each islet (https://www.surgery.wisc.edu/system/assets/353/Islet_Viability_Assessment_by_Fl._Microscopy.pdf?1264176101).

A reliable method to rapidly determine the viability of isolated islet cells is necessary in studies on transplantation, cryopreservation, insulin release, and biosynthesis. Functional assays to determine insulin content or release and morphological assays including histochemistry or electron microscopy are too time-consuming and expensive for routine viability assays (Bank, 1987).

Therefore, vital staining is preferred to perform visual counting of live or dead cells to other methods. Chromogenic inclusion dyes such as neutral red and exclusion dyes such as trypan blue are commonly used to assay the membrane integrity of numerous cell types. However, these dyes have a limited usefulness in the viability evaluation of cell structures (Bank, 1987). In fact, both staining methods are incapable of visualizing both live and dead cells simultaneously within a whole islet.

The yellow tetrazole of the C,N-diphenyl-N'-4,5-dimethyl thiazol 2 yl tetrazolium bromide (MTT) dye is a colorimetric assay that has been suggested to be an indirect estimation of mitochondrial oxidative processes of living cells. Considering the unique metabolic feature of the test, it appears to be particularly suitable for assessing the function of the beta cell, capable of modulating its glycolytic and oxidative rates as a function of extracellular glucose concentrations. The MTT assay thus provides a convenient tool for the rapid assessment of beta cell metabolism and viability (Janjic and Wollheim, 1992).

The most commonly used assay to assess islet viability in humans and large animals is the fluorescein diacetate and propidium iodide (FDA/PI) double staining assay (Woolcott et al., 2012). FDA has been used as a fluorometric assay of cell viability (Bank, 1987). FDA functions as an inclusion dye, being a colorless, non-polar ester that passes through the plasma membrane and becomes hydrolyzed by non-specific cellular esterases to produce fluorescein, resulting in a strong green fluorescence. PI, an exclusion dye, is polar and stains only the late apoptotic and necrotic cultured cells (Foglieni et al., 2001), because their membranes are permeable to the dye; once inside the cell, it fluoresces orange/red when bound to nucleic acids. Under a fluorescence microscope, the approximate volume fraction of cells stained of each color is visually assessed.
FDA/PI is a quick, easy, and cheap assay and therefore very convenient. However, the stainings are not specific to only islets but stain all cells (Islam, 2015). In fact, some results showed that FDA was unsatisfactory due to the high extracellular fluorescence (Bank, 1988). Further, different test conditions and the experience of the operator may influence the outcomes and the final assessment of the viability (Islam, 2015).

An alternative approach is the combination of acridine orange/propidium iodide (AO/PI), which allows the observation of intact and damaged cells simultaneously. The AO/PI assay is also capable of simultaneously visualizing live and dead cells in intact islets (Bank, 1988).

Efforts are still ongoing to find alternative staining methods, such as dyes that are responsive to metabolic activity (e.g. tetrazolium salts, ATP, ADP/ATP, oxygen consumption rate) and/or mitochondrial membrane potential (Islam, 2015).

1.5.5. Islet culture

Optimal culture conditions are imperative to allow the recovery of the islets from the isolation procedure while maintaining the tridimensional structure of the clusters and preventing islet loss (Islam, 2015). Factors like the temperature and media composition are crucial as well as the need for supplementation of the medium with growth factors and other compounds that allow long-term culture (Islam, 2015).

In humans, rodents, caprines, dogs and pigs, islets are typically cultured under standard conditions at 37°C with 5% CO_2 (Carter et al., 2009; Hani et al., 2010; Vakhshiteh et al., 2013).

The selection of the appropriate culture medium has been shown to depend on the animal source of the islets (Islam, 2015).

Connaught Medical Research Laboratory 1066 (CMRL) is the most widely used base medium for islet culture in humans. It is a chemically defined medium developed in the late 1950's by Connaught Medical Research Laboratories. CMRL-1066 was designed initially for use with L-strain cells in unsupplemented culture. It has been used to maintain monolayer growth of permanent cell lines for years without protein supplementation. Although developed for use in serum free cell culture, CMRL-1066 can be supplemented with serum and used to support the growth of many types of cells (www.sigmaaldrich.com).

Other base media used for clinical islets transplantation include the Ham's F10, the Medium 199 and RPMI-1640 (Islam, 2015).

Roswell Park Memorial Institute medium, commonly referred to as RPMI medium, is a medium used in cell and tissue culture. It has traditionally been used for growth of human lymphoid cells. This medium contains a great deal of phosphate and is formulated for use in a 5% carbon dioxide atmosphere. RPMI-1640 has traditionally been used for the serum-free expansion of human lymphoid cells. RPMI-1640 uses a bicarbonate buffering system and differs from most mammalian cell culture media in its typical pH 8 formulation. Properly supplemented with serum or an adequate serum replacement, RPMI-1640 allows the cultivation of many cell types, especially human T/B-lymphocytes, bone marrow cells and hybridoma cells (Moore et al., 1967).

M199 is used as preferred medium for the culture of porcine islets (Jin et al., 2014) while RPMI-1640 is the preferred medium for islet culture in rodents (Carter et al., 2009) and caprines (Hani et al., 2010; Vakhshiteh et al., 2013). Both RPMI-1640 and CMRL have been also used to culture dog islets (Vrabelova et al., 2014; Woolcott et al., 2012).

Regardless of whichever base medium is employed, supplementation of the medium seems to be a routine practice. Because serum contains many components that have a beneficial effect on cell survival, animal serum such as fetal calf serum is traditionally added to culture media in experimental settings (Islam, 2015), although fibroblast proliferation might be better controlled in serum-free media (Hani et al., 2010). Several studies have also tested the administration of various growth factors and compounds in order to improve the culture conditions of islets; these included e.g. glutamine, human albumin, insulin, and sericin (Daoud et al., 2010). In fact, the presence of L-glutamine and theophylline in media was found to positively influence the process of insulin release (Hani et al., 2010). Supplementation with antibiotics like penicillin and streptomycin reduces the risk of contamination (Carter et al., 2009).
Islet isolation exposes the islets to a variety of cellular stresses and disrupts the cell-matrix relationship, which may potentially lead to apoptosis (Daoud et al., 2010). For these reasons, new supplementation factors, such as antioxidants that preserve islet integrity and viability of islets during culture, have been tested (Gaber and Fraga, 2004; Bottino et al., 2004).

The concentration of glucose in the medium is another key factor which may influence both the apoptosis rate and islet viability. Rodent islets are routinely cultured in media containing 11mM of glucose (Carter et al., 2009). Lower glucose concentrations may reduce islet insulin content and downregulate key genes related to glucose metabolism (Islam, 2015) whereas extended exposure to high glucose might cause toxicity (Carter et al., 2009). In humans, short exposure to high-glucose (16.8mM) may cause islet apoptosis while prolonged exposure to glucose concentration higher than 6mM may already suppress insulin biosynthesis (Fred et al., 2010; Eizirik et al., 1992).

The capacity to maintain islets in culture may vary among different species. Rodent islets can preserve glucose sensitivity for at least one week in culture when the medium is changed frequently (Carter et al., 2009). Viability and purity of caprine islet preparations seem to

increase after a short-term culture of five to seven days, possibly because non-viable islets are removed before plating and culture conditions may privilege the survival of endocrine over exocrine tissue. On the other hand, prolonged islet culture may result in diminished responsiveness to glucose challenges and cause endocrine tissue loss. In addition, central necrosis of islets is more frequently induced by long-term culture (Hani et al., 2010).

Plating density is another important factor, which plays a critical role in islet culture. High numbers of islets per volume of culture medium may induce necrosis as a consequence of cell damage and competition for nutrients (Islam, 2015). Caprine islets are usually kept for *in vitro* maintenance at a ratio of 100 islets per 1ml of medium (Hani et al., 2010). Islets cultured at high surface densities in standard T-flasks also exhibit low viable tissue recovery, viability, and potency, possibly due to anoxic conditions. These effects have been prevented by culturing islets in gas-permeable devices, which increase oxygen availability (Papas et al., 2005).

One of the drawbacks of growing cells *in vitro* using conventional tissue culture techniques is that the cells rest on plastic rather than on their natural biological support. Several new biomaterials have been developed to improve islet viability during culture and provide an extracellular matrix that recreates conditions similar to the native islet microenvironment (Daoud et al., 2010). Some of the most promising solutions include the use of Extracelular Matrix Pre-coated plates (ECM), islet encapsulation, scaffolding techniques, and bioreactors (Islam, 2015). For instance, the ECM is a natural support formed by a complex network of macromolecules such as collagen, laminin and fibronectin, which have been used to coat tissue culture plastic and have shown to improve cell attachment and growth (Novamed-ECM).

1.5.6. Functionality

For the final assessment of the quality of an islet preparation, it is essential to characterize the functionality of the islets (Islam, 2015). A fundamental property of pancreatic islets is their capacity to regulate the release of insulin and other hormones in direct response to changes in extracellular glucose concentration. This ability defines islet function since insulin is produced and released in the body only from islet beta cells. Glucagon, somatostatin, and

other peptides are also produced by islet cells, but in smaller amounts and they are therefore more difficult to be detected (Carter et al., 2009).
When performing functional tests, a high degree of islet purity is crucial. In fact, the presence of exocrine tissue in the islet preparation can influence the secretory response of the islets. For islet transplantation studies, it is however important to mention that *in vivo* function cannot always be predicted from *in vitro* data (Hani et al., 2010).

The standard method used to measure islet function *in vitro* is the glucose-stimulated insulin secretion test (GSIS) (Islam, 2015).
The GSIS test consists of cultivating islets at "low" glucose concentration (typically near 3mM), to measure the amount of insulin secreted into the media under "basal" or "unstimulated" conditions. Stimulated insulin release is then measured by exposing islets to a higher glucose concentration such as 11.1mM (half-maximal) or >28mM (maximal) (Carter et al., 2009). The purpose of alternating "basal" or "unstimulated" conditions with stimulated ones is to assess that the islets in the preparation are able to respond properly to their natural stimulus, i.e. to increase and also to decrease insulin secretion according to the respective changes in glucose concentration (Islam, 2015).

Other available techniques to measure islet function include the analysis of the calcium concentration (Ca^{2+}) in response to changes in extracellular glucose concentration.
In addition, islet functionality may be evaluated by the measurement of the insulin and C-peptide content after extraction of both peptides from isolated islets and quantification in the supernatants through immunoassay tests (Carter et al., 2009). As an alternative, islet function may be indirectly assessed by assaying mitochondrial activity since mitochondria plays a crucial role for glucose-stimulated insulin secretion.

1.6. Islet isolation in cats

A review of the literature shows very few reports on the successful isolation of pancreatic islets in cats.

A first study was conducted in 1998 by Hatchell et al. who described a collagenase-based method for the isolation of feline islets. However, the purpose of this study was to determine if the subretinal space in cats would provide an immune privilege for allografts of islets of

Langerhans, and did not exhaustively describe the isolation method by itself (Hatchell et al., 1998).
Nonetheless, the authors reported that the pancreas was removed immediately after euthanasia inflated via the pancreatic duct with cold collagenase (1,5 mg/ml in HBSS, Serva) and incubated for 40 minutes at 38ºC in a shaking water-bath. The purification of the digestate was performed with filtration and manual-handpicking techniques (without dithizone staining). The isolated islets were than incubated at 32ºC in medium 199 supplemented with 10% fetal bovine serum, 10mmol/L nicotinamide, antibiotics, and antimycotics for 24 hours before transplantation. No information on the viability of the islets was given, though islets survived the transplantation and were revascularized in the subretinal space of healthy cats (Hatchell et al., 1998).

In 2006, a subsequent study from the same group aimed at investigating a method for isolating a sufficient number of islets able to restore normoglycaemia in pancreatectomized cats, following transplantation into the subretinal space. In addition to the previously published method, Maeno et al. showed that supplementation of the culture medium with 10% FBS increased islet viability compared to the supplementation with 0,25% BSA. In this study, islet viability was assessed by fluorescein diacetate/ethidium bromide staining. The purity of the islet clusters was evaluated after staining with dithizone and confirmed by immunohistochemistry. Islet yield was of only 300 islets per pancreas and purity was very low since most islets were surrounded by acinar cells. Interestingly, electron microscopy of intact cat pancreas disclosed very little basement membrane-like material surrounding the islets. Collagenous layers surrounding islet cells have been observed in most of the other species (such as dogs and rats), for which methods have been established to obtain sufficient yields of acinar cell-free islets after collagenase digestion. In view of this consideration, Maeno concluded that the paucity of collagen present in feline pancreas may be responsible for the difficulty of obtaining pure islets free of acinar cells in cats (Maeno et al., 2006).

A few years later, our group published a study where six different collagenase-based methods for the isolation of feline islets were compared. The study's aim was to establish a method that would enhance purity of isolated islets, facilitating future studies on the pathophysiology of diabetes. Pancreas perfusion was performed immediately after euthanasia with *ex situ* injections of the enzymatic solution across the pancreatic capsule. Injections were preferred

because the authors reported that the identification and cannulation of the pancreatic duct was very difficult in cats and in most cases not feasible. Zini and colleagues compared single and double course of collagenase digestions to pancreas digestion with Accutase, a cell detachment solution of proteolytic and collagenolytic enzymes and with a mix of Collagenase and Accutase. Purification was performed with the Ficoll density centrifugation media and by filtration. Based on morphometric analysis, there were no differences in terms of purity between the procedure one or two courses of collagenase digestion, respectively. However, isolation of pancreatic islets by single and double course of collagenase digestions was considered superior to the other two enzymatic solutions tested. Even though islet isolations were performed according to standardized protocols, the ratio between pancreatic islets and exocrine tissue varied widely among cats, suggesting that individual differences were present among cats. Further, because the large majority of retrieved islets were surrounded by residual exocrine pancreas, the average purity of isolates was not considered satisfactory (Zini et al., 2009).

1.7. Aim of the study

Previous experiments by our and other groups showed that the isolation of pancreatic islets in cats cannot be considered satisfactory until now (Zini et al., 2009; Maeno et al., 2006). If viable, pure and functional islets can be isolated in cats, *in vitro* studies can be performed, allowing the investigation of specific mechanisms of beta cell dysfunction and loss occurring in feline diabetes.

The specific aims of our study were therefore:

1) To improve islet isolation protocols in cats and to increase islet yield, purity and viability.
2) To test the functionality of isolated cat islets.

The approach described in this thesis is based on the method established for mice and described by Li et al., 2009. Each step of the original protocol was adapted in order to optimize the method for the use in cats.

2. MATERIALS AND METHODS

2.1. Donor animals

Nine cats were used as donors for pancreatic islet isolation between March 2014 and March 2015. The harvesting of *pancreata* was performed in the Clinic for Small Animal Internal Medicine of the Vetsuisse Faculty, University of Zurich, after cats' euthanasia because of end-stage disorders unrelated to this study. Cats with pancreatitis or other pancreatic diseases, with systemic infection or undergoing immunosuppressive therapy were excluded from the study.

Four cats were males (1 neutered; 2 intact; 1 unknown), three were females (2 neutered; 1 unknown) and in two cases the sex was unknown. In those cases where the cats' age was known, they ranged between 4 and 17 years old (median age: 8 years); six cats were European short-hair, one was a Maine Coon and the breed of two cats was unknown **(Tab. 1)**.

Table 1: Donor characteristics: Age, breed, sex, neutering status and reason for euthanasia of the nine cats included in the study

Donor characteristics	**Cat #**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Age (years)	17	15	~3-5	4	N.a.	N.a.	4	N.a.	5
Breed	EC	MC	EC	EC	EC	N.a.	EC	N.a.	EC
Sex	M	M	M	M	F	N.a.	F	N.a.	F
Neutering status	Yes	No	N.a.	No	N.a	N.a.	Yes	N.a.	Yes
Reason for euthanasia	Mega Eso-phagus	Dys-pnea	Hit by car	Hit by car	Neuro-logic disease	N.a.	Neo-plasia	N.a.	Lym-phoma

N.a. - Not available; EC - European Common; MC - Maine Coon; M - Male; F - Female

2.2. Anatomical study in cats

Initial anatomical studies were performed during *post mortem* examinations in a pilot study, in five extra cats, in order to identify the most practical and effective technique to perfuse the cats' pancreas. Three different methods were tested and pancreatic perfusion was performed

by injecting 2% Evans Blue solution with a Venofix Safety system (25G, Braun, Melsungen, Germany):

a) The common biliary duct - *in situ*: after clamping the major papilla on the duodenum wall to block the retrograde liquid flow to the duodenum, the Venofix Safety system was inserted into the common biliary duct to the position where the hepatic duct and the cystic duct merge into the common duct, following a protocol used in mice (Li et al., 2009). Evans Blue dye was then perfused with a 10ml syringe until the complete distension of the pancreas was achieved.

b) The pancreatic duct in the area of the major papilla - *in situ*: after clamping the common biliary duct, duodenum and pylorus, the Venofix Safety system was introduced from the duodenal lumen side in the area of the major papilla pointing towards the common biliary duct. Evans Blue dye was then perfused with 10ml syringes until the complete distension of the pancreas was achieved.

c) The pancreatic duct, after excision of the pancreas - *ex situ*: after clamping the common biliary duct, duodenum and pylorus, the pancreas was sharply dissected from the duodenum and stomach, immersed in Hanks' Balanced Salt solution (HBSS; GIBCO, Paisley, Scotland) with 1,26mM $CaCl_2$ and 0,49mM $MgCl_2$-$6H_2O$ at 4ºC and transported to the laboratory. After being washed with cold (4ºC) HBSS, the pancreas was placed on a sterile cooled plastic tray and cut approximately in the middle of the body in two halves. Angiocatheters (18G, 22G or 24G; Terumo, NJ, USA) or polyethylene tubings (0,64mm of diameter, Intramedic, USA) of different sizes were tested and inserted into the main pancreatic duct on each half of the pancreas. Blue Evans solution was perfused via the pancreatic duct until the complete distension of the pancreas was achieved.

2.3. Feline islet isolation

All information regarding each of the nine islet isolations are described in the following paragraphs and are summarized in Table A (Annex).

2.3.1. Pancreas perfusion

Immediately after confirmation of cardiac arrest, cats were placed in dorsal recumbency and the abdomen was aseptically prepared. A ventral midline laparotomy was performed and the pancreas was localized.

In order to reduce the warm ischemic time (the time that the organ remains at body temperature after its blood supply has been interrupted), the pancreas was chilled at 4-8ºC by moistening the organ with cold NaCl 0,9% (Fresenius Kabi AG, Bad Homburg, Germany) and by direct contact with cold packs (case #8 and #9).

Based on the results described in the section "Anatomical study in cats" (b), the enzymatic solution (see the next paragraph for details) was perfused *in situ* via the pancreatic duct in the area of the major papilla. Three different volumes of enzymatic solution (50ml, 80ml and 100ml) were tested to assess the minimum volume needed to achieve the complete distension of the pancreas. After the entire pancreas was greatly distended, a total pancreatectomy was performed, the excised pancreas was immersed in ice-cold enzyme solution (4ºC) and transported on ice to the laboratory for immediate processing.

When the perfusion of the pancreatic duct was not possible through the major papilla, the cannulation of the duct was performed *ex situ* (see "Anatomical study in cats" (c)). In this case (case #8), the pancreas was then cleaned from vascular, fat and connective tissue with sterile surgical instruments.

2.3.2. Pancreas digestion

Pancreata were perfused with an enzymatic solution containing 50mg/ml of collagenase type IV (Worthington, NJ, USA) of which two different lots were tested (A – 250U/mg and B – 310U/mg), 10mg/ml of DNase I (>2000U/mg, Roche, Germany) and 1% Hepes (1M, Gibco, UK) in HBSS. To define the optimal conditions for the pancreas digestion, different pancreas sizes and digestion times were tested. After the perfusion, the pancreas was cut either into ~1.5 cm pieces (each immersed in 2ml of the enzyme solution; cases #1-7) or used as entire organ and immersed in 50ml of the enzyme solution (case #9). Digestion times varied between 15 and 60-min in a water-bath at 37°C. The digestion of the entire pancreas was performed in similar conditions until approximately 50% of islets were free from acinar tissue, based on guidelines from human islet isolation protocol (Lehmann et al., 2007).

Pancreatic beta cells have a high zinc content and staining with dithizone (DTZ), a zinc-chelating agent, is a quick and simple way to identify insulin-producing beta-cells in a mixed-cell pancreas preparation. Therefore, every 10', 1ml of pancreas digestate was recovered, stained with 140µl of DTZ (Sigma-Aldrich, Austria) solution (see below) and observed under the light microscope (100x, Axiovert 40 CFI, ZEISS). Islets were categorized according to the percentage of acinar cells attached to it in three different groups (≤50%, 50-75%, ≥75%).

To prepare the DTZ working solution, 50mg of DTZ powder was reconstituted with 10ml of Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, USA) and 30ml of phosphate-buffer saline (PBS). The saturated solution was filtered through a 0,45µm filter.

In one case (cat #9), in addition to the incubation in a water bath at 37°C, the enzymatic digestion of the entire pancreas was supported by mechanical disruption via manual shaking at 5-min intervals.

The time from the beginning of the incubation at 37°C until the final step of the digestion with the transfer of the samples at 4°C was recorded for each cat. After the digestion was stopped, samples of pancreas digestate were manually shaken 30-40 times to obtain a more homogenous solution. Ice cold quenching buffer (35ml per 2ml of digestate; solution of HBSS with 2,4% HEPES 1M and 0,5% BSA) was added to the samples, tubes were inverted 3 times, and centrifuged at 550x g for 2 minutes at 4ºC. The recovered pellet was resuspended in 20ml of ice-cold quenching buffer and centrifuged again under the same conditions described above.

2.3.3. Islet purification

The pellet of pancreas digestate was resuspended again in 10ml of ice cold quenching buffer and the solution was filtered through a 500µm strainer (136cm, SEFAR PETEX, Heiden, Switzerland) using a 16G/10cm metallic needle (Delvo, Switzerland) attached to a 10ml syringe. Filtrations were performed one or two times and results were compared.

Islet purification was then performed by the so-called "filtration method" consisting of a filtration step through a 70µm cell strainer (Falcon, USA) up to three times. Purified islets were then tapped from the surface of the strainer into 10ml of RPMI-1640 culture media (11% fetal calf serum, 1,14% streptomycin-penicillin, 0,11% gentamycin, 0,11% fungison, 1,14% Glutamax; Gibco, Paisley, Scotland) containing 11mM of glucose.

After isolation and purification, islets were hand-picked with a 20µl pipette, resuspended in fresh culture medium RPMI-1640 and incubated at 37°C/5% CO_2 for 2 hours to recover from the stress of the isolation process. Islet hand-picking was performed by using either a dissecting microscope (50x amplification, Stemi 2000-C ZEISS) or a normal light microscope (100x amplification, Axiovert 40 CFI ZEISS).

In order to distinguish islets from aggregates of exocrine acinar cells or other cell types and helping the hand-picking process, purified islet preparations were stained with either 0,5% Neutral Red (NR, Sigma, USA) or with 1,25% DTZ as previously described. Neutral Red is a dye commonly used for staining in histology and also used as specific and nontoxic stain of pancreatic islets. Briefly, islets were filtered and collected on the surface of a 70µm strainer, then exposed to 0,5% NR (two different times were tested: 10 sec or 3 min) and washed with Krebs Ringer Buffer (KRB, Tab. 4). Stained islets were tapped into 6ml of fresh KRB and observed under the microscope.

2.4. Assessment of islet viability and quality

2.4.1. Viability

Samples of purified islets were placed in Petri dishes (35mm x 10mm, Corning, USA) with 3ml of culture media (RPMI-1640) and islet viability was evaluated with four viability staining methods.

In the first method, Trypan Blue (0,4% TB, Sigma, USA), a vital stain used to selectively color dead cells in blue, was used. Briefly, 0,5ml of 0,4% TB and 0,3ml of HBSS were added to 0,2ml of the original islet suspension and incubated for 5 - 15min at room temperature. Two samples of islet suspension (10µl each) were loaded into each of the two grids of a haemocytometer. Both viable and dead islets were counted under a light microscope (50x, Axiovert 40 CFI, ZEISS) and the proportion of dead cells was expressed as a percentage of the total for each experiment.

The second method relies on dimethylthiazol (MTT, 50ng/ml, Sigma, USA) which is a yellow tetrazole dye and which is reduced to purple formazan in living cells; hence, MTT can also be used to assess the viability of islets. Briefly, islets were incubated in culture medium with 1% MTT for 15, 30 or 40min and viability was assessed using an inverted microscope (50x and 100x, Axiovert 40 CFI, ZEISS). As negative control, islets were first incubated in 30%

Hydroxide-peroxide solution (H_2O_2, Sigma, Germany) for 30min, to assure the death of all cells, and then stained with MTT.

Third, islet viability was assessed with the vital stain Neutral Red at concentrations of 0,01% and 0,5%, as previously described. Neutral Red is incorporated into the lysosomes of living cells, therefore, the lack of dye uptake corresponds to the loss of cell viability.

As fourth method, a double fluorescence-based method that combines the use of the cell permeable esterase-substrate fluorescein diacetate (FDA, Life Technologies, North America) with the cell impermeant nucleic acid stain propidium iodide (PI, Life Technologies, North America) was tested. Living cells actively convert the non-fluorescent FDA into the green fluorescent compound "fluorescein" as a sign of viability while nucleus membrane-compromised cells will fluoresce red as a sign of cell death (Jones et al., 1985). On the day of the isolation, islets were stained with 1,25% DTZ to be distinguished from non-islet structures. DTZ-positive islets were then hand-picked and washed in a Petri dish containing 3ml of fresh culture medium for 24 h. The day after the isolation, islets were stained with 20µl PI (1:10) followed by 20µl of FDA (1:100) for 30 seconds and then washed in a 35mm Petri dish containing 2.0ml of PBS. At least five separate fields were visualized and photographed with an inverted microscope with fluorescent capability (Axiovert 200M, ZEISS), utilizing a long-distance magnification of 200x and the filter block for fluorescein (~ 530 nm) and rhodamine (>600 nm). Average viability was assessed for each islet based on the principle that FDA-positive cells are dead and stain red while PI-positive cells are viable and stain green. The average viability of each islet was assessed based on the percentage of viable *versus* dead cells and included in the following categories (**Tab. 2**; https://www.surgery.wisc.edu/system /assets/353/Islet_Viability_Assessment_by_Fl._Microscopy.pdf?1264176101):

Table 2: Categories of islets' average viability, according to the percentage of islet cells that are viable within each islet

Percentage of green vs. red cells within each islet	Average viability (%)
Few or no cells are green and the majority are red	Non-viable
Approx. 75% of the cells are red	25%
Approx. 50% of the cells are red	50%
Approx. 25% of the cells are red	75%
Few or no cells are red	100%

Red cells = dead cells; Green cells = live cells

(https://www.surgery.wisc.edu/system/assets/353/Islet_Viability_Assessment_by_Fl._Microscopy.pdf?1264176101)

2.4.2. Morphology, yield and purity

Islet samples were stained with 1,25% DTZ and observed under a light microscope. External morphology (shape, structure and color), yield (defined as the total number of islets obtained from a pancreas) and purity of islets (defined as the percentage of free and the percentage of trapped islets to the total number of islets counted) were assessed by visual inspection in samples of purified islets before handpicking (case #9).

A 35x10mm cell culture dish (w/2mm grid, Thermo Scientific, NY) and an eyepiece with a 1mm scale (10µm grid; E-PI 10x/20, ZEISS) were used to determine the islet size. Islets were divided into seven categories according to their diameter **(Tab. 3)**; islets smaller than 50µm were not included in the manual count.

Table 3: Islets' size intervals

Islets' diameter (µm)
50 – 100µm
101 - 150µm
151 - 200µm
201 - 250µm
251 - 300µm
301 - 350µm
> 350µm

Note: Islets larger than 150µm are considered big islets

2.5. Islet culture and functionality assessment

On the day of the isolation, a pre-defined number of islets (Case #7: N= 4; Case #9: N= 15) were randomly distributed and cultured in 500µl of RPMI-1640 medium containing 11mM glucose in 24-well ECM-coated plates (Novamed, Israel) for 48h at 37°C and 5% CO_2. ECM tissue culture dishes are coated with a naturally produced basement membrane-like extracellular matrix that mimics the cells natural extracellular matrix framework. Cells in contact with ECM attach rapidly and have been shown to have prolonged life span and better functional characteristics compared to cells cultured in common culture dishes (Personal communication: Novamed, Israel). Before any test was conducted, islets were allowed to settle down and adhere to the plate's well, and medium was not changed during the first 48 hours.

2.5.1. Glucose stimulated insulin secretion test (GSIS)

Glucose-stimulated insulin secretion was determined by performing a GSIS test 48h after isolation. First, culture medium was removed at baseline and then stored at -80°C. Then, 500µl of Krebs Ringer Buffer 1X solution **(Tab. 4)** with 2,8mM glucose was added to the islets and incubated for 30min at 37°C. The solution was discharged and islets were resuspended in 500µl Krebs-Ringer 1X solution containing low (2,8mM) glucose concentration and incubated for 1h at 37°C. The Krebs solution was then collected and spinned down at 3000rpm for 5min. Supernatants were stored at -80°C until insulin measurement was assayed (insulin secretion at low glucose concentration). Next, islets were incubated in 500µl of Krebs Ringer Buffer 1X solution containing high glucose concentration (16,7 mM) for 1h at 37°C. As before, supernatants were stored at -80°C (insulin secretion at high glucose concentration). Finally, 500µl of 0.18M HCl in 70% EtOH solution was added to the islets and incubated for 2h at room temperature. Supernatants were kept at -80°C for the measurement of the total insulin content of the islet at the end of the test.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

In order to assess the usefulness of the Feline Insulin ELISA assay (Mercodia, Uppsala, Sweden) for the measurement of insulin in supernatants from feline islet cultures, recovery and linearity of dilution experiments were performed as follows. Recovery experiments were used to determine if the assay is affected by interfering factors contained in the islet culture medium. High concentrations of insulin (Calibrator 5) were spiked into different volumes of

either Ringer Krebs, RPMI medium or Calibrator 0 (kit diluent) samples and then analyzed for recovery.

Table 4: Krebs Ringer Buffer 1X

Substance	Concentration
NaCl	115mM
KCl	472mM
$CaCl_2 \cdot 2H_2O$	2,56mM
KH_2PO_4	1,2mM
$MgSO_4 \cdot 7H_2O$	1,2mM

Stock solution: 6,72g NaCl + 0,35g KCl + 0,164g KH_2PO_4 + 0,3g $MgSO_4 \cdot 7H_2O$; fill with distilled water until 800ml and correct the pH to 7,4. Add 2,5ml of $CaCl_2 \cdot 2H_2O$ 1M and fill until 1L with distilled water.
Working solution: 100ml stock solution + 1ml Hepes 1M + 0,5g BSA.

The results were expressed as a percentage of insulin recovered. Recoveries between 80-120% were considered acceptable, demonstrating no significant diluent interference with the assay.

The linearity of the assay determines the extent to which the dose-response of the analyte is linear in a particular diluent. In order to test this, dilution series of Calibrator 5 (at 1:2; 1:4; 1:8; 1:16) were generated using RPMI medium or Calibrator 0. The results were expressed as a percent observed from expected. Values between 80-120% show good assay linearity.

Intra-assay precision was determined from the mean coefficients of variation (CV) of two measurements of the same sample within one assay. Mean CVs below 10% were considered acceptable, between 10% and 15% marginally acceptable and above 15% unacceptable.

3. RESULTS

3.1. Anatomical study in cats

All five anatomical studies were performed *post mortem* in previously refrigerated animals. Due to *post mortem* degenerative changes, the elasticity and texture of the tissues were inferior compared to those of freshly euthanized animals.

The perfusion *in situ* through the common biliary duct could not be performed in these cats. While in mice, the common biliary duct can be accessed through the abdominal cavity with no need to open the thoracic cavity (Li et al., 2009), the access to the common biliary duct through the abdominal cavity and thus the perfusion of the pancreas was, in most cases, not possible in cats.

The perfusion *in situ* through the pancreatic duct at the area of the major papilla was found to be the most successful of the three methods tested to perfuse the pancreas in cats. Although the major papilla was usually surrounded by a big layer of fibrous tissue, the access to this area and the perfusion of the pancreas resulted in a pancreas distension of approximately 80% of the total pancreas volume in all four cases tested with this method. The body and the head of the pancreas were always the first parts being distended, followed by the tail **(Fig. 1)**.

Figure 1: Cat pancreas perfused with 2% Evans blue dye

1. Head; 2. Body; 3. Tail; 4. Duodenum

Pancreas perfusion *ex situ* through the insertion of an angiocatheter or a polyethylene tubing into the pancreatic duct was attempted in one cat, although with no success. From a more careful examination of the path and size of the pancreatic duct in all cats tested, we observed that the diameter of the duct progressively decreases and that the duct produces ramification of much smaller diameter sizes towards the tail and the head of the organ. In addition, there seemed to be a high inter-individual variety among cats in regards to the duct diameter that made the selection of the correct size of the catheter or tube difficult. These specific anatomical characteristics could not be assessed macroscopically. Dissection of the pancreas along the pancreatic duct under a light microscope did not help to predict the size of the duct and of the potentially most suitable catheter size. Nevertheless, because angiocatheters are harder than polyethylene tubings, they are easily manipulated. The size of catheters that fitted the pancreatic duct best was the 24G, i.e. the smallest tested here.

3.2. Feline islet isolation

Details regarding each of the nine islet isolations are described in the following paragraphs and are summarized in **Table A** (Annex). Viability, quality and purity of the islet preparations were not systematically and quantitatively assessed in all cases; hence, they are reported as personal observations of single cases.

3.2.1. Pancreas perfusion

In general, the warm ischemia time decreased with the experience of the operator in performing laparotomy in cats. Further chilling of the pancreas by direct contact with cold packs and with an intra-abdominal infusion of cold NaCl 0,9% was performed only in two cases (cat #8 and #9). The independent effect of a reduced warm ischemia time on the final quality of the islet preparation could not be assessed in these two cases, because other changes were made to the protocol at the same time which could have influenced the final outcomes; however, based on observations made in other species (Hani et al., 2010; Kim et al., 2009), it is believed that the cooling process of the pancreas will positively influence the final outcome of the islet isolation.

In eight of the nine cases (case #1-7 and #9), the perfusion of the pancreas was successfully achieved through the major papilla. Three *pancreata* were perfused with 50ml, four with 80ml and one with 100ml. Pancreas distension was insufficient with the 50ml volume while distension reached approximately 90% with the larger volumes of 80 and 100ml, respectively.

No major differences in terms of distension of the pancreas were observed between the 80 and the 100ml volume. However, in all cases, soon after pancreatectomy, the enzyme solution started to leak out of the organ and the overall pancreas distension decreased markedly. In general, the quality of the islet preparation increased with increased pancreas distension during the perfusion.

In case #8, perfusion of the pancreatic duct was not possible through the major papilla. Therefore, pancreatectomy was followed by the duct cannulation *ex situ* through an angiocatheter (24 G). As observed during the anatomical studies, the ductal cannulation was extremely difficult to be accomplished with this method and despite many attempts, only approximately one half of the pancreas was perfused.

3.2.2. Pancreas digestion

i) Type of enzyme solution

Perfusion was performed using the Collagenase type IV enzyme solution from Worthington - two cases with 250mg/ml of lot A (250U/mg), seven cases with 50mg/ml of lot B (310U/mg). Using the same digestion protocol in the cases #1-7, we observed that Collagenase type IV from lot A seemed to produce a digestate containing less exocrine tissue than the one produced after digestion wit lot B. By using lot B, however, the number of free *versus* trapped islets was higher compared to lot A. Nevertheless, more isolations with either one or the other lot should be performed to assess the inter-lot variability in the efficiency of islet isolation.

ii) Size of the pancreas

In one case (#9) in which the entire pancreas was digested in a single container, a higher yield of free islets was observed compared to the other cases (cases #1- 7) in which the pancreas was digested after being cut into small pieces.

iii) Time of digestion

In seven cases, pieces from the same pancreas were submitted to different fixed digestion time points (15, 30, 40 and 60min). In these cases, we observed that, generally, the highest yield and purity of islets were obtained at 30 and 40min of digestion. Only in one case (cat #1), a longer digestion (60min) resulted in a higher quality of digestate. In general, pancreas digestion for more than 40min appeared to result in loss of membrane integrity.

Digestion of the entire pancreas in case #9 was stopped after 81min when approximately 50% of islets were free from acinar tissue **(Fig. 2)**. Although the digestion time was much longer compared to the times necessary for digestion in the other cases, the overall purity of the islet preparation was higher. The assessment of the digestate quality for this case is summarized in **Table 5**.

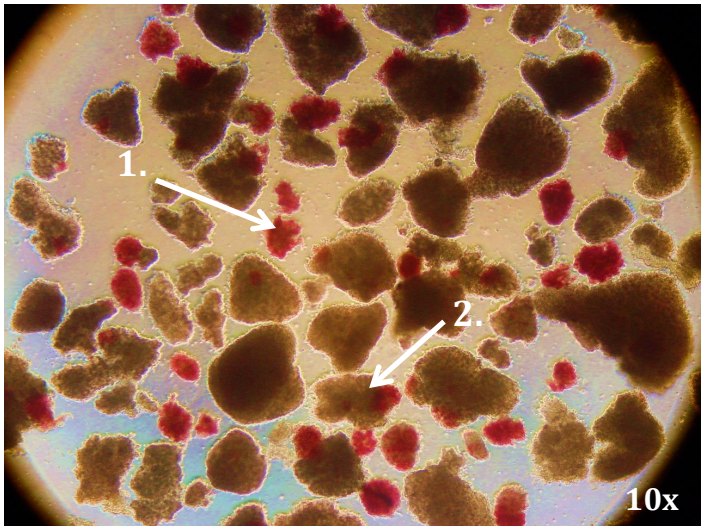


Figure 2: Islets were stained red by DTZ in digested pancreatic tissue after 81 minutes of digestion (Case #9)

1. Free islets; 2. Exocrine tissue was not stained and appeared dark-yellow

Table 5: Assessment of the pancreas digestate quality (Case #9)

Time of digestion	Total # of islets	% of acinar cells attached to the islet		
		≤ 50%	50-75%	≥ 75%
48'	4	3		1
	6	5		1
	5	2	2	1
55'	4	2	2	
	2	1	1	
	4	2	2	
68'	5	3	1	1
	5	4		1
	3	1	2	
75'	7	1	3	3
81'	6	4	1	1
	3	1	1	1
	7	4		3

Note: At the indicated time points (approximately every 10'), a sample of pancreas digestate was recovered, stained with 140µl of DTZ and observed under the microscope (100x) - islets are included in three different categories according to the percentage of acinar tissue surrounding each islet.

iv) Mechanical disruption

Mechanical disruption, consisting in manual shaking of the pancreas at 5min intervals has been reported to assist in breaking down the interlobular fibrous tissue to release the islets (Gray et al., 2004). This procedure was only performed in cat #9 in which the pancreas was processed as entire organ and resulted in a more homogenous and fluid digestate product. In other words, the entire pancreas tissue was digested and disaggregated and the size of the floating particles was the same compared to those obtained in the other cases.

3.2.3. Islet purification

In the first four isolations, pancreas digestate was filtered one time through a 500µm strainer to separate the digestate from undigested tissue. As the quality of the final islet preparation was not considered satisfactory (the ratio between non-islet tissue/islets was too high), a second filtration step through a 500µm strainer was tested in the two cases #5 and 6. Since no improvements were observed in terms of purity of the digestate, a single filtration through a 500µm strainer was performed in the following cases #7 and #9.

In all cases, additional filtration of the digestate through a 70µm strainer was used as purification method. Multiple filtrations were tested in order to improve the final purity of islets preparations (one filtration tested in cases #1-3, #5 and #9); two filtrations tested in cases #6 and #7; three filtrations tested in cases #4 and #6). We observed that multiple filtration steps seem to be useful because the higher the number of filtration steps through the 70µm strainer, the higher the proportion of islets *versus* exocrine tissue and the higher the degree of islet purity of the final preparation.

In six of the eight cases, a 2nd purification step by the handpicking technique was necessary to obtain islets preparation of almost 100% purity. The use of a dissecting microscope with a bottom light source was found to be more adequate compared to the use of a light microscope for this purpose. In the first four cases, handpicking was performed based on islet morphology and on the experience achieved by performing islet handpicking in mice, therefore no islet-specific staining was applied beforehand. However, we found that cat islets were extremely difficult to be distinguished from exocrine structures both in terms of shape and color (**Fig. 3 - A**). For this reason, islet handpicking was performed after islet-specific staining in the

following cases. Because Neutral Red (0.5%; **Fig. 3 - B**; case #7) stained all structures red and was not specific for islets, the use of 1,25% DTZ was preferred **(Fig. 5)**.

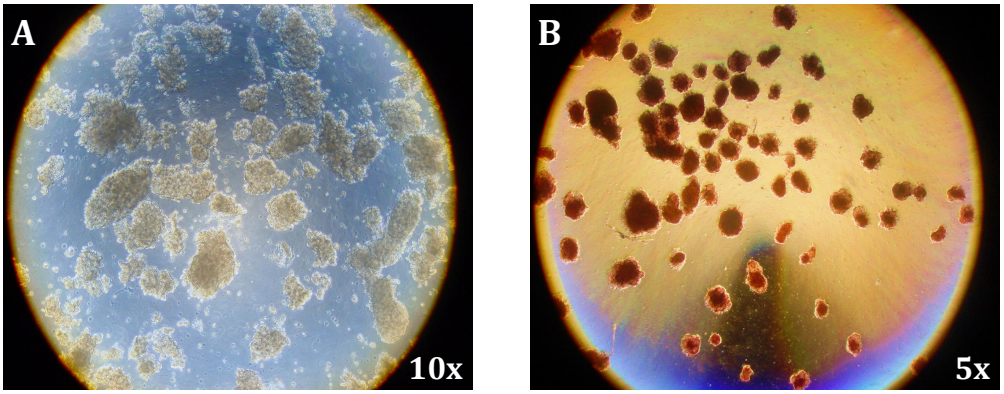


Figure 3: Islet preparation after the first purification step

A – Not-stained: islets cannot be distinguished from non-islet structures

B – Stained with 0,5% Neutral Red: all structures in the islet preparation are stained red because the staining is not specific for islets

3.3. Assessment of islet viability and quality

3.3.1. Viability

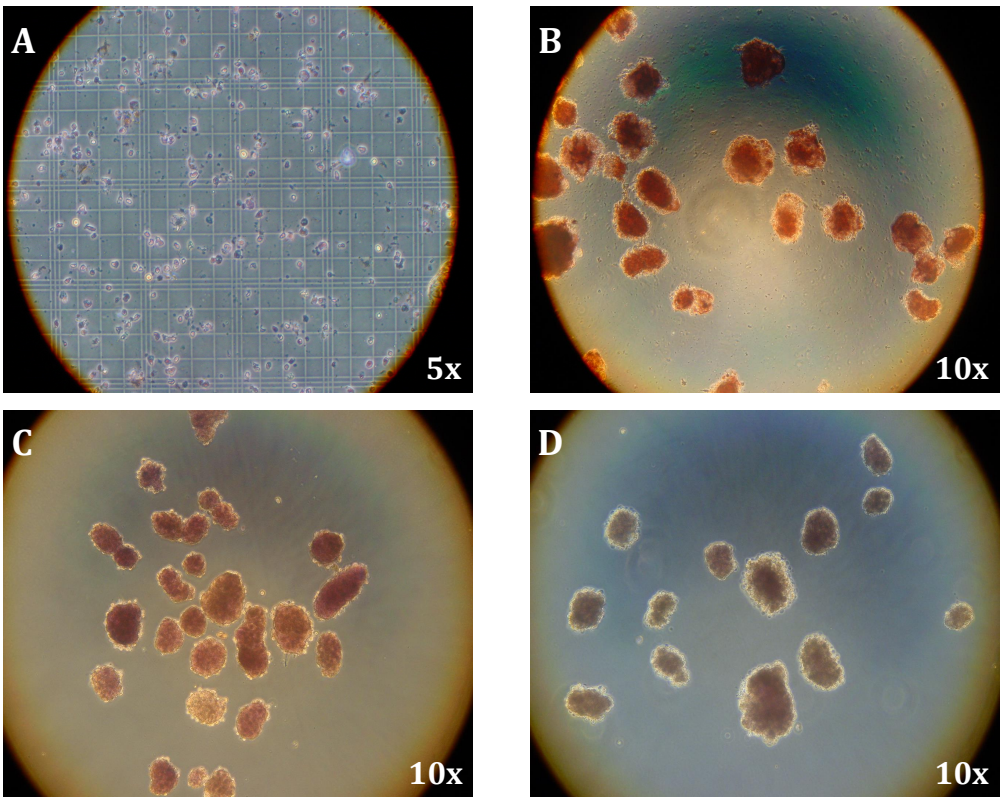
To assess the viability of purified islets, four different staining methods were tested: Trypan Blue (in case #2 and #3), MTT (in cases # 3-7), Neutral Red (in case #6) and the fluorescent staining FDA/PI (in case #9); in some cases, more than one staining technique were tested in parallel.

Staining of islets with 0,4% Trypan Blue allowed to distinguish between alive (blue) and dead (white) structures; unfortunately, a real quantification of the number of dead *versus* alive islets was not possible because islets disaggregated by sliding into the hemocytometer chamber **(Fig. 4 -A)**.

With the 1% MTT assay, alive structures stained in intense purple, whereas dead particles were not stained. Islet viability was checked after 15, 30 and 40min of incubation under a light microscope **(Fig. 4 - C)**. As a negative control, cell death was induced by incubation with 30% H_2O_2 for 30 minutes followed by MTT staining **(Fig. 4 - D)**. Although the MTT staining distinguished between alive *versus* dead islets, it did not allow to assess the percentage of viable cells within each islet. Besides this, because islets have a tridimensional structure, dead cells which were not stained (and probably the minority – around 10%) were not visible under the light microscope. Overall the islets viability assessed with this method was of 90%.

Neutral Red dye at the concentration of both 0.01% and 0.5% was not considered a reliable staining method for the assessment of islet viability since it stained all the structures from the islet preparation in red and was therefore unspecific for islet *versus* non-islet material **(Fig. 4 - B)**.

Because islets are aggregates of cells, it is desirable to assess the correct percentage of cells within an individual islet that are alive or dead in order to have a more accurate information about the effect of the digestion procedure on the islet health. The FDA/PI fluorescent staining allowed to distinguish between alive (green) and dead (red) cells within each islet. As a general observation, islet integrity and shape were preserved 24h after the isolation. 1,25% DTZ staining, which was routinely performed on the day of isolation to distinguish between islets and non-islets particles, did not interfere with the quality of the fluorescent staining performed 24h after islet isolation **(Fig. 4 - E, F)**. In case #9, the average islet viability assessed with the FDA/PI fluorescent staining was of 75%.



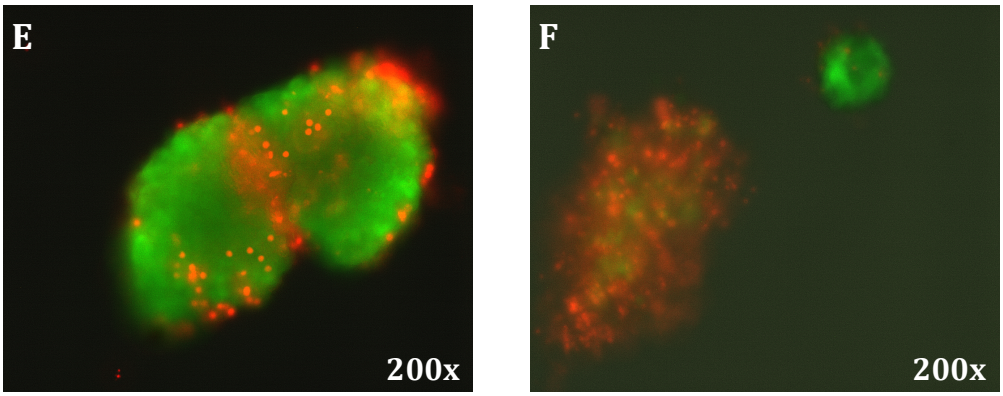


Figure 4: Islet viability assessment - four different stainings were tested

A – Islets stained with 0,4% Trypan Blue (white - alive cells; blue - dead cells)

B – Islets stained with 0,5% Neutral Red (red - alive cells; not-red - dead cells)

C – Islets stained with 1% MTT (purple - alive cells)

D – Islets previously killed with H_2O_2 (used as negative control) *stained with 1% MTT (not-purple - dead cells)*

E, F – Islets stained with FDA/PI fluorescent stainings (green - alive cells; red - dead cells)

3.3.2. Morphology, yield and purity

On the day of isolation and after the first purification step, the majority of the DTZ-positive islets appeared to have an irregular shape and to be free from acinar tissue **(Fig. 5 - A)**.

Culture of islets in standard medium for the following 24h allowed the reversal of the DTZ staining. Once unstained, most structures appeared to have an islet-like, egg-shape form; however, an additional DTZ staining (24h after isolation), revealed that only a low percentage (around 10%) of these structure were really pure islets free of acinar tissue. The majority of the islets which, on the day of the isolation, appeared to be free from the exocrine tissue, seemed to be trapped by some acinar cells when assessed 24 h after isolation. In fact, the non-islet components of the preparation seemed to attach to the islets during the incubation time, decreasing the degree of purity of the islets and the general quality of islet preparation **(Fig. 5 - B)**. Handpicking on the day of isolation was therefore necessary to assure and preserve the quality and purity of islet preparations.

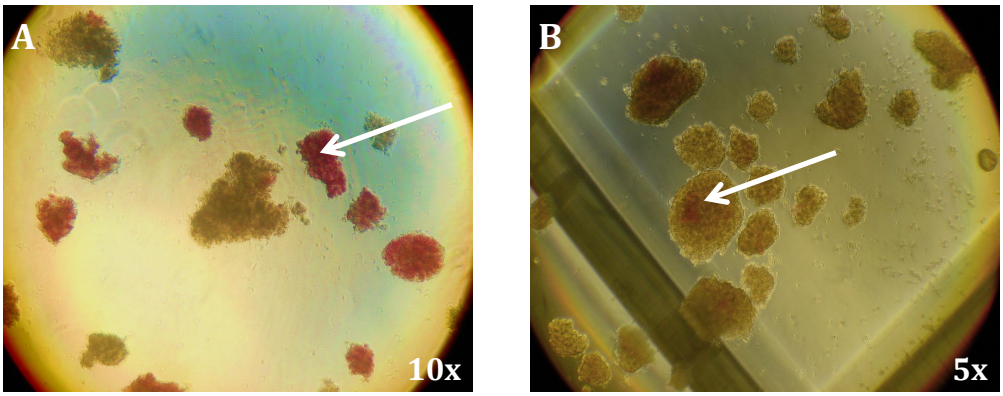


Figure 5: Preparations with DTZ-stained islets (arrow)

A – Day of isolation; B – 24h after isolation

The range of size distribution were between 50 to 100µm. Islets larger than 100µm were not observed.

3.4. Summary of achieved results

In summary, islet preparation with the highest quality was obtained when 80ml of collagenase type IV (Worthington) were perfused through the pancreatic duct at the site of the major papilla. The enzymatic digestion of the entire pancreas was performed in a water-bath at 37°C in combination with mechanical disruption until approximately 50% of islets were free from acinar tissue (after DTZ staining).

3.5. Islet culture and functionality assessment

In standard culture plates, islets were free-floating and did not adhere to the surface of the plate, but they adhered well to the bottom of ECM plates after 48h of culture **(Fig. 6)**. When attached to the ECM culture plate, islets presented a white bright color, whereas when they were floating they kept the yellow color they had before.

Figure 6: After 48h of culture, islets that adhered to the bottom of ECM plates presented a white bright color (arrow)

In our study, a plating density of 15 islets (case #9) per well resulted in an increased number of adherent and viable islets. We also found that the islets attached better to the plates when the medium was not changed within the first 48h.

The glucose stimulated insulin secretion (GSIS) assay used to assess the islet functionality was successfully performed, following the guidelines from mouse islet isolation (Carter et al., 2009).
However, the reliability of the measurement of insulin levels in supernatants from islet culture using a feline insulin ELISA kit (Mercodia, Uppsala, Sweden) was not considered sufficient. This kit is commonly used for direct quantitative determination of insulin levels in feline serum or plasma samples. The interference of culture media with the ELISA assay was assessed by performing recovery and linearity of dilution experiments.

In the first ELISA, the relative recovery (measured insulin concentration in samples diluted with Ringer Krebs *versus* expected values of Calibrator 5) was 105% when samples were diluted 1:2 but decreased to 74% in 1:8 dilutions **(Tab. 6)**.

Table 6: Analysis of the recovery of insulin calibrator 5 spiked to Ringer Krebs assessed using a feline Insulin ELISA kit

Compound	Conc1	Conc2	Mean	Corrected for dilution factor		Mean conc	SD	CV %	Recovery %
Ringer Krebs - BLANK	-45.0	-46.9	-46	0.00		0.00	1.34	#DIV/0!	
Ringer Krebs + Cal 5 1:1	775.5	804.5	790.0	790.0	100.0	790.0	20.6	2.6	
Ringer Krebs + Cal 5 1:2	423.2	405	414.1	828.2	50.0	414.1	12.9	3.1	105
Ringer Krebs + Cal 5 1:8	73.2	72.0	72.6	580.9	12.5	72.6	0.8	1.1	74

In order to improve the accuracy of the measurements and following the company's recommendations, known amounts of insulin were not diluted in series; instead each dilution was made directly from the Calibrator 5 vial. Also, plastic polypropylene tubes were used instead of Borosilicate glass tubes (Corning Incorporated, NY).

Despite these changes, the relative recovery of insulin Calibrator 5 spiked to RPMI-1640 or Calibrator 0 was poor and was considered to be below acceptable criteria (recoveries between 80-120% were considered acceptable, demonstrating no significant diluent interference with the assay) **(Tab. 7)**.

Table 7: Analysis of the Recovery of insulin Calibrator 5 spiked to RPMI-1640, Ringer Krebs and Calibrator 0 assessed using a feline Insulin ELISA kit (Catalog #10-1233-01; Mercodia)

Diluent	Average Recovery %	Range
Calibrator 0	72.0	58-84 %
Ringer Krebs	89.2	102-74 %
RPMI-1640	75.4	69-89 %

The recovery was also not acceptable in the linearity assay and results were similar for both samples diluted with Calibrator 0 and with culture medium **(Fig. 7)**. Therefore, insulin detection was not affected by the difference between diluent used for preparation.
However, low absorbance of the calibrator curve was observed in four out of six ELISAs **(Fig. 8 and 9)**. This factor may have influenced the accuracy of the assay in detecting insulin.

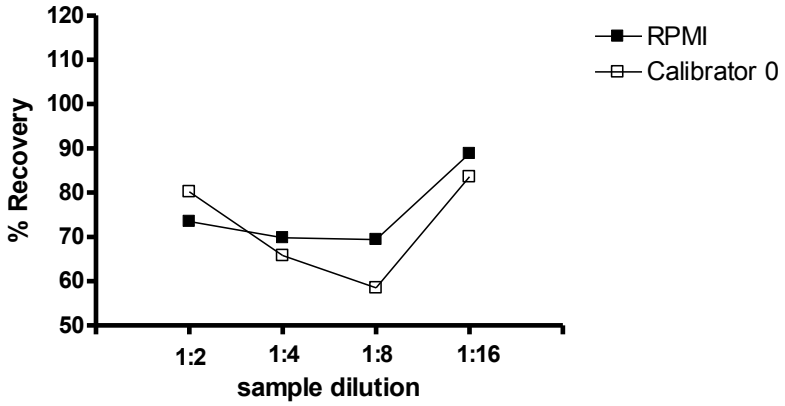


Figure 7: Assay Linearity. Spiked insulin Calibrator 5 samples were diluted with either Calibrator 0 or RPMI-1640 medium and assayed using a feline Insulin ELISA Kit. The percent recovery of samples diluted with Calibrator 0 had a range of 58.5-83.6%. Linearity of dilution was also found to be low (69.4-88.8%) with sampled diluted with RPMI-1640 medium

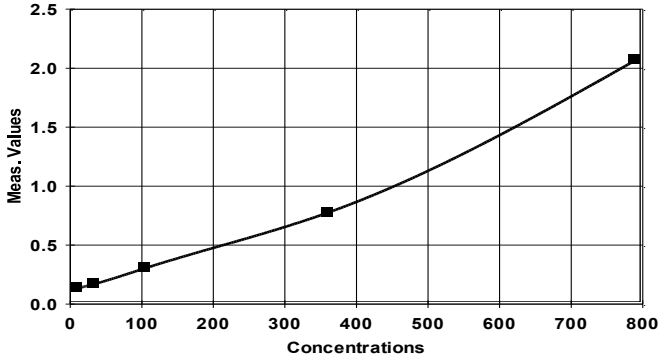


Figure 8: Graphs of normal absorbance of the standard curve (calibrators' concentrations: 1 – 9,2ng/L; 2 – 34ng/L; 3 – 105ng/L; 4 – 360ng/L; 5 – 790ng/L) – the

concentration of feline insulin is obtained by computerized data reduction of the absorbance for the calibrators, except Calibrator 0, *versus* the concentration using cubic spline

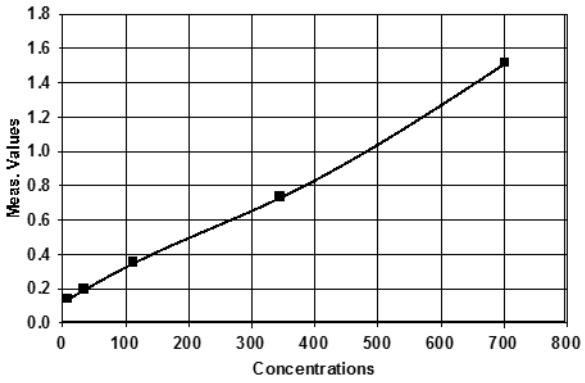


Figure 9: Graphs of low absorbance of the standard curve

Potential technical reasons for low absorbance could be due to: a) too low intensity of the plate reader lamp; b) a wavelength used to read absorbance different than 450nm; c) a low amplitude of the plate shaker; d) the use of non-calibrated pipettes resulting in low sample / calibrator volume; e) insufficient incubation time; f) wrong dilution of the enzyme conjugate solution and soak included in the wash procedure, as described in the Troubleshooting manual from Feline Insulin ELISA kit, Mercodia. All potential reasons were carefully checked and none was found to be the source of the error. Also, low absorbance was not an operator-related problem since it was observed when assays were performed by different operators. Finally, the absorbance of the calibrator curve was usually in the normal range when different ELISA kits, namely the mouse insulin kit from the same company, were performed by the same operators.

4. DISCUSSION

Diabetes mellitus is a common endocrinopathy in cats whose prevalence has highly increased in the last years (Sparkes et al., 2015). Approaches that are designated at gaining further knowledge on the pathophysiology of the disease are of pivotal importance. Among all, research isolated islets has greatly contributed to the understanding of diabetes causing mechanisms in humans and rodents. In this context, we and others have attempted to establish a method for the isolation of pancreatic islets in cats in the past years (Maeno et al., 2006; Zini et al., 2009). However, because the quantity and quality of islets obtained after the isolation were scarce, results were considered unsatisfactory and not comparable to those in other species, in particular rodents. Nevertheless, the potential advantages of using isolated islets for the study of the pathophysiology of feline diabetes have motivated us to pursue the research in this field. In this study, we improved the previously described methods for the isolation of islets in cats by enhancing not only the yield and the purity, but also the viability and functionality of the isolated islets.

Islet isolation is a complex procedure whose successful accomplishment depends on many factors. Therefore, the standardization of the criteria for the inclusion of pancreas donors into a study is essential. In humans, pancreas donors are standardized for having a similar age (average: 40.9 ± 1.4 years), cause of death, body mass index (BMI between 25 and 30 is preferred) and the absence of diseases, which may directly or indirectly affect the pancreas (O'Gorman et al., 2005).

The cases included in the present study were recruited from a population of cats that were euthanized at the Clinic for Small Animal of the University of Zurich for a variety of non-standardized conditions. Collagenase-based islet isolation was performed in nine cat *pancreata* following the guidelines of an optimized method commonly used in mice (Li et al., 2009). Several changes were applied to adapt each step of the original protocol to the specific characteristics of the cat pancreas.

Cats suffering of any pancreatic disorders or systemic infection or under immunosuppressive therapy were excluded from this study, however, major differences namely in the age, body condition score and time of hospitalization before euthanasia, were present between donors.

In addition, harvesting of the pancreas was legally allowed only in cats which were non-heart beating at the time of perfusion. It is known that *pancreata* from brain-dead organ donors

may produce lower yields of less viable islets due to donor haemodynamic instability, high doses of inotropic support and inflammatory reactivity that predispose for additional organ injury. For these reasons, although it may not be ethically accepted at the time being, working with heart beating animals should be considered in further studies (Woolcott et al., 2012; Kin et al., 2009).

This and the other factors mentioned above have been already demonstrated to affect the quality of isolated islets in other species (Balamurugan et al., 2014; O'Gormon et al., 2005) and may, therefore, have significantly influenced the outcomes of our study. Moreover, the unpredictability of cases recruitment and the urgency of processing the pancreas immediately after euthanasia, have greatly limited the recruitment of cats in this study that was in general difficult and resulted in a small sample size. To improve the recruitment strategy, further studies would benefit from being conducted at more than one center or clinic and from a 24 hours availability of a team of researchers.

A classical procedure of islet isolation includes three main steps: pancreas perfusion; pancreas digestion and islet purification.

4.1. Pancreas perfusion

It has been proved that cold preservation of the pancreas before perfusion protects islets from the deleterious effect of prolonged warm ischemic time (Vrabelova et al., 2014).

In order to simplify the donor organ harvesting and to limit the expenses of the study, we opted for the surface cooling of the pancreas (#8 and #9). However, the effect of a reduced warm ischemia time on the final quality of the islet preparation could not be assessed in these two cases, because other changes were also made to the protocol, which obviously could have influenced the final outcomes. Moreover, due to logistic constraints, warm ischemic periods of more than 15min occurred in a few cases though, some authors have suggested that such period should not exceed 10min (Hani et al., 2010). The influence of these aspects on the final quality of isolated islets needs to be further investigated in cats.

In agreement with previously published studies in humans (Kin et al., 2007), we confirmed that the pancreas perfusion through the pancreatic duct leads to a more efficacious diffusion of the enzymatic solution into pancreatic *acini* and to a more selective digestion of the exocrine pancreas compared to other methods. Although the major papilla can be easily identified on the cat duodenum, the *in situ* cannulation of the pancreatic duct through the

papilla may be challenging and considerable expertise is required to increase the probability of success of this technique. In humans, a pancreatectomy is usually performed prior to the cannulation procedure and only after one or more catheters are inserted into the main pancreatic duct, either in the middle or in the extremities of the body (Seiji et al., 1999).

In cats, the *ex situ* cannulation of the pancreatic duct was difficult and, in most cases, not possible, as already reported in previous studies (Maeno et al., 2006; Zini et al., 2009). However, this technique offers some advantages over the *in situ* cannulation such as that the pancreas can be conserved into preservation buffer up to some hours after euthanasia and its perfusion can be performed in the lab, eventually using the Ricordi chamber for the digestion step (Hakim et al., 2010).

Alternative methods, such as the cannulation of the common bile duct (CBD), the elective method used in rodents (Li et al., 2009), was not possible in cats, most likely because the CBD is very short in this species and the access to it is hampered by the diaphragm and the ribs.

4.2. Pancreas digestion

Based on the method from Li *et al*., we used the Collagenase type IV from Worthington, a crude enzyme derived from C. histolyticum. Crude enzymes contain heterogeneous proteases and enzymes (Kin et al., 2007), cellular debris, pigments and endotoxins (Hyder, 2005) in addition to collagenase, and may, therefore, be considered enzymes of low purity. The Collagenase type IV from Worthington has, however, a low inter-lot variability probably because of the low collagenase and protease activity (around 10%) on a "per milligram" basis (Personal communication: Vitacyte, LLC). Nonetheless, the purity (defined as the percentage of free *versus* trapped islets to the total number of islets counted) of the islets isolated with this enzyme was low and the majority of islets were surrounded by acinar cells. These findings indicate that enzymes with different characteristics may be more appropriate when working with cat pancreas.

The last generation of collagenase from Vitacyte (type I+II) associated with Neutral Protease have shown to facilitate a more cost-effective isolation with the use of less amount of enzyme, while preserving cellular and metabolic integrity and increasing the purity of humans islets (Caballero-Corbalán et al., 2009). This may, therefore, represent a potential collagenase candidate to be used in cats. However, as stated before, the choice of the right enzyme should be taken according to the species-specific structure and anatomy of the pancreas. Therefore, a

histologic characterization of islet architecture and composition, similar to what is available in other species (Steiner et al., 2010; Wieckorek et al., 1998), should also be performed in cats.

The variability in enzyme potency is an obstacle to the use of collagenases in islet isolation practice (Barnett et al., 2005). In mice and in other laboratory animals, the activity of every single lot of collagenase is tested and the optimal digestion time is set in pilot studies. However, because in large animals or humans, each islet isolation is an experiment on its own, this kind of testing cannot be performed. Therefore, to control the collagenase activity during the digestion process, digestate samples are stained with dithizone (DTZ) at regular intervals. Digestion is stopped when the percentage of acinar cells attached to the islets is below 50%. Using this method, digestion can be well controlled and the quality of islet preparations obtained with different collagenase lots can be compared (protocol DTZ). Because cats as humans present with a high inter-individual variability in the amount of collagen surrounding the islets and in the ratio between pancreatic islets and exocrine tissue (Zini et al., 2009), a dynamic control of the digestion process is to be preferred to fixed digestion times. In our study, the introduction of a DTZ-based control of the digestion step has greatly improved the purity of pancreas digestates.

In our study we obtained higher yields of free islets when the pancreas was not cut in pieces before digestion. In humans, the traditional method of digestion inside the Ricordi chamber is performed with the organ intact; however it is customary nowadays to cut the pancreas into blocks before placing it into the chamber. However, there is little data confirming a real advantage of this modification on the regular method (Hakim et al., 2010).

The digestion time may also influence the outcome of islet isolation. In our study, the best ratio between yield and purity of islets were obtained after 30 and 40min of digestion. Longer digestion times might sometimes be necessary to obtain higher islet purity. In fact, the longer is the digestion of the connective tissue which covers the pancreas, the better is the access of the collagenase to the parenchyma and the more islets are free from acinar tissue. However, higher purity may results in lower islet yield as a consequence of overdigestion.

Finally, manual or automatic shaking has been shown to increase the breaking down of the interlobular fibrous tissue and the islet yield in several species (Li et al., 2009, Vrabelova et

al., 2014; Hani et al., 2010; Gray et al., 2004). According to our observations in cats, manual shaking has greatly helped to mechanically disrupt the pancreas during digestion and resulted in higher islet yields.

4.3. Islet purification

Islet purification is a key step to obtain pure (i.e. free from acinar tissue) islet preparations to be used for islet transplantation and *in vitro* studies.

In this study, islet preparations were purified by filtration. The degree of purity obtained with this method was however considered not acceptable, meaning that the large majority of retrieved islets (80-85%) were surrounded by residual exocrine tissue.

A second purification step is often needed to increase islet purity prior to culture (Carter et al., 2009). Islets handpicking has, therefore, been applied to improve the degree of purity and ensure the achievement of qualitatively-high islet preparations to be used for culture and functional assessment in our cat studies. For the handpicking process as well as for the evaluation of the morphology and degree of islet purity in cats, islet-specific staining revealed to be essential. Mouse islets are easily identified by their semi-opaque color in comparison to the relatively transparent exocrine tissue (Islam, 2015). Different from mice, specific beta cell staining is required to distinguish cat islets from the rest of the pancreas tissue. The same seems to happen in larger animals and humans (Gray et al., 1983). While mice islets have a central core of beta cells (representing 60-80% of the cells of the islet), and a layer of other endocrine cells surrounding the core, the islets of the domestic cat have a centralized area of alpha cells with surrounding beta cells and a few solitary omega cells (Steiner et al., 2010). We hypothetized that such differences in islet architecture and endocrine cell composition may explain the different color and aspect of islets between species, as well as the general problem to isolate islets in cats.

4.4. Viability

Among the different staining methods tested in this study, the fluorescent staining FDA/PI resulted to be the method which provided the most reliable information about the viability of single cells within the tridimensional islet structure and was not influenced by the staining with DTZ.

In fact, because pure islets could only be identified after DTZ staining followed by

handpicking, potential interferences of DTZ with viability stainings had to be tested. Based on the guidelines from the University of Wisconsin for the assessment of islet viability by FDA/PI, we assessed an average islet viability of 75% (https://www.surgery.wisc.edu/system/assets/353/Islet_Viability_Assessment_by_Fl._Microscopy.pdf?1264176101). This result was satisfactory, although no major conclusions about the general viability of our islet preparations can be based on only one case.

An alternative method to DTZ is to stain beta cells with the zinc specific fluorescent dye Newport green (NG). NG was tested (Stork and Li, 2006) in a double fluorescent staining protocol together with PI and proved to be a valid alternative to DTZ in human islets. NG staining was tested in one cat islet preparation, however, the assessment of the overall viability of islets was not performed using a confocal microscope in this study.

4.5. Morphology, yield and purity

In this study, freshly isolated cat islets appeared as dark yellow spheroids with irregular edges. The majority of islets had a diameter between 50 and 100μm. Similar islet size distribution has been reported in mongrel dogs (Lakey et al., 1998). Because distribution with higher peak in the category of islets with diameter less than 100μm has been associated with islet fragmentation in dogs (Alejandro et al., 1990) and rats (Morini et al., 2006), it is likely that the isolation method used in the present study might also be associated with islet fragmentation (Wolcoot et al., 2012).

In our study, only feline large-sized islets (≥150μm) presented a darker central core. A possible explanation for this fact may be due to a greater diffusion barrier to oxygen and nutrients in large-sized islets (Macgregor et al., 2006; Williams et al., 2009). In general these islets were not attaching or were detached after some hours from the bottom of the ECM plate, indicating that they were not viable islets (Personal communication: Novamed).

Because the goal of our study was to establish a protocol to obtain viable, free and functional islets, we prioritized quality and purity to quantity. For this reason, we did not quantified the total islet yield per cat pancreas.

Cleavage through the interface between endocrine and exocrine cells, i.e. the structural integration of the islet in the exocrine pancreas, is a prerequisite for obtaining both clean and structurally intact islets (van Deijnen et al., 1992). Therefore, the abundance of collagen surrounding the islets is of pivotal importance for the successful isolation of islets by

collagenase digestion (van Deijnen et al., 1992).

Following this theory, Maeno et al. (2006) attributed the difficulty to isolate pure islets in cats to the minimal amount of basement membrane-like material surrounding the cat islet. Van Suylichem et al. (1995) demonstrated that it is not the amount of collagen in the septa but the one in the rest of the pancreas, mainly located between the *acini* that seems to determine the dissociation of the pancreatic tissue. In other words, the higher the amount of collagen around the islets, the higher the islet purity obtained despite of the total amount of collagen. Hence, we speculated that the difficulty encountered in the present study to isolate pure islets free from the surrounding acinar tissue, may be due to the relative low quantity of collagen which surrounds feline islets. However, to the best of our knowledge, there is only very little information available about the quantity of collagen surrounding the islets in cats (Maeno et al., 2006). Once again and, as mentioned before, a more detailed characterization of the distribution and content of collagen in the cat pancreas is required to further understand the process of islet isolation in this specie.

4.6. Islet culture and functionality

Feline islets were cultured under the standard conditions used in mice (Li et al., 2009) and medium was not changed during the first 48h after the isolation in order to allow the complete recovery of the islets from the stress experienced during the isolation and purification steps. After 48h, unattached islets were washed off and considered as non-viable. The higher the degree of purity of the islets, the higher the survival rate under culture conditions. In humans, the preparation and purification of islets are as important as the quality of the culture medium because it has been shown that exocrine cell contamination greatly hampers islet cell survival in culture (Schmied et al., 2000).

In cats as in mice and humans, islets free-float in the medium and do not attach well to the uncoated dishes, whereas when plated on ECM culture plates, islets attach to the dish within 48h. Working with islets attached to wells is easier and prevents the loss of islets during the medium change or functional tests. We have also noticed that, similarly to what happens with mouse islets, survival of cat islets seems to be prolonged by the culture on ECM plates compared to uncoated plates (personal observation).

Within a certain range (4 to 15 islets per 500µm of medium), we observed that the higher the plating density, the more successful the adhesion of islets to the bottom of the plate and

consequently their survival under culture conditions. In rodents, the optimal islet density is four islets per square centimeter (Carter et al., 2009). According to the literature, caprine islets are usually kept for *in vitro* maintenance in a ratio of 100 islets per 1ml of medium (Hani et al., 2010).
In our study, pure islets were not all devoted at being cultured but were also used to establish the best method for the purification and the viability assessment. Thus, the maximum plating density was of only 15 islets per 500µl of medium. Further studies should consider to increase the number of islets per ml of medium to define the optimal plating density in cats.

The standard culture medium used in this study was selected based on previous studies conducted in mice and contained a glucose concentration of 11mM (Islam, 2015). This supra-physiological concentration might be needed to provide new energy for the recovery of islets from the stress of the isolation procedure. However, considering the physiologic glucose levels in cats, prolonged islet culture at 11mM of glucose may simulate more a hyperglycemia-like or even glucotoxic-like state than physiological conditions in cats.
Several studies have shown that chronic elevation of blood glucose concentrations in both humans and experimental animal models leads to beta cell dysfunction in terms of insulin secretion and insulin synthesis (Moran et al., 1997; Harmon et al., 1999; Robertson et al., 1999). Therefore, further studies are required to identify the medium that best provides enough nutrients and energy to feline islets without influencing their functionality.

We also attempted to assess islet functionality by a glucose stimulated insulin secretion (GSIS) assay as described in mice (Islam, 2015). The measurement of insulin levels in supernatants from islet culture was performed using a feline insulin ELISA kit (Mercodia, Uppsala, Sweden), a test which is commonly used for direct quantitative determination of insulin levels in feline serum or plasma samples. The interference of culture media with the ELISA assay was assessed by performing recovery and linearity of dilution experiments. Although we excluded any interference of culture medium with the feline ELISA assay, the accuracy of the assay in detecting insulin in supernatants from feline islet cultures was considered not acceptable. The functional potency of purified islets is measured by calculating their secretion index as the ratio of stimulated to basal insulin secretion normalized by the insulin content. While insulin levels in plasma and serum samples are usually in the low range and are measured undiluted in the assay, supernatants collected after

GSIS tests contain insulin levels that are above the upper limit of detection and, thus, need to be diluted up to 1000 times. For this reason, because we observed that the sensitivity of the feline insulin ELISA was higher in the very low range of insulin concentration but decreased in the higher range, the accuracy of the assay in detecting insulin was considered to be below acceptable criteria.

In summary, to the best of our knowledge, no studies are available in the literature on *in vitro* culture of feline islets. We are, therefore, the first group who succeeded in culturing feline islets at a high degree of purity, for five days. However, the culturing conditions used to generate these preliminary data will need to be further characterized and improved, including a full functional characterization of the islets.

4.7. Conclusion

In conclusion, islet isolation was successfully accomplished in eight cases. The preparation with the highest islet quality was obtained by perfusing the pancreas with 80ml of Collagenase type IV (Worthington) through the pancreatic duct at the site of the major papilla. The enzymatic digestion of the intact organ was associated with mechanical disruption and controlled by frequent DTZ staining. Purification was performed by a first filtration step followed by handpicking. Purified islets were then plated on ECM plates and cultured for 48h before a functionality test (GSIS) was performed.

For the first time, we were able to isolate and culture feline islets with high degree of viability and purity. However, as the islet yield and the percentage of pure *versus* the total number of isolated islets was low compared to data on other species, further studies are undoubtedly required to improve the procedure of islet isolation in cats.

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6. ANNEX

Table A: Summary of the characteristics of the nine cases studied

		CASE #							
		1	2	3	4	5	6	7	8	9
PERFUSION	**Method of cold-ischemia time**	X	X	X	X	X	X	X	cold NaCl + cold packs	cold NaCl + cold packs
	Volume needed to perfuse pancreas	50ml	50ml	50ml	100ml	80ml	80ml	80ml	X	80ml
	Via of perfusion	major papilla	major papilla	major papilla	major papilla	major papilla	major papilla	major papilla	ductal cannulation	major papilla
DIGESTION	**Type of enzyme**	Worthington lot 1	Worthington lot 1	Worthington lot 2	Worthington lot 2	Worthington lot 2	Worthington lot 2	Worthington lot 2	Worthington lot 2	Worthington lot 2
	Size of the pancreas	cutted	cutted	cutted	cutted	cutted	cutted	cutted	entire	entire
	Time of digestion	Fixed (30', 40', 60')	Fixed (40', 60', 80')	Fixed (30', 40', 60')	Fixed (30', 40, 60')	Fixed (30', 40', 60')	Fixed (30', 40', 60')	Fixed (15', 30', 40')		Variable (DTZ, until 90')
	Mechanical disruption	X	X	X	X	X	X			shaking
PURIFICATION	**Number of 500µm passages**	1x	1x	1x	1x	2x	2x	1x		1x
	Purification method	Filtration 1x70µm	Filtration 1x70µm	Filtration 1x70µm	Filtration 3x70µm	Filtration 1x70µm	Filtration 2-3x70µm	Filtration 2x70µm		Filtration 3x70µm +Biocoll
	2nd Purification step/used staining	X	X	X	X	X	X	NR 0,5%		DTZ Sigma
VIABILITY	**Viability assessment/used staining**	X	TB	TB + MTT	MTT	MTT	NR + MTT	MTT		a)DTZ b)FDA/PI
	H2O2 negative control/used staining	X	X	X	X	MTT control	MTT control	MTT control		X
PURITY	**Morphology, yield, purity assessment/used staining**	DTZ Millipore	DTZ Millipore	DTZ Millipore	X	DTZ Millipore	DTZ Millipore	DTZ Sigma		DTZ Sigma
	H2O2 negative control/used staining	X	X	X	X	DTZ Millipore control	DTZ Millipore control	X		X
	Quantitative /qualitaive assessment using DTZ	X	X	X	X	X	X	X		✔
FUNCTIONA-LITY	**Islet culture for GSIS**	X	X	X	X	X	X	ECM plate (7-13islets)		ECM plate (15islets)
	GSIS test	X	X	X	X	X	X	✔		✔

7. LIST OF ABBREVIATIONS

AO/PI acridine orange/propidium iodide

BMI body mass index

C clostridium

CBD common biliar duct

CHO chinese hamster ovary

CMRL connaugh medical research laboratories

CV coefficient of variation

DM diabetes mellitus

DTZ dithizone

ECM extracellular matrix pre-coated plates

FDA/PI fluorescein diacetate/propidium iodide

FDM feline diabetes mellitus

GSIS glucose-stimulated insulin sensitivity test

IEQ islet equivalent

KRB krebs ringer buffer

MR magnetic retraction

MTF mammalian tissue free

MTT tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

NG newport green

NR neutral red

RIA radioimmunoassay

T1DM type 1 diabetes mellitus

T2DM type 2 diabetes mellitus

TB trypan blue

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